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Award Number: DAMD17-95-C-5071

TITLE: Poly (Methyl Methacrylate) (PMMA) and Polyactic Acid Nanoparticles as Adjuvants for Peroral Vaccines

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REPORT DATE: June 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1999	Final (26 Sep 95 - 25 May 99)

4. TITLE AND SUBTITLE
Poly (Methyl Methacrylate) (PMMA) and Polyactic Acid Nanoparticles as Adjuvants for Peroral Vaccines

5. FUNDING NUMBERS
DAMD17-95-C-5071

6. AUTHOR(S)
Jorg Kreuter, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Chemotherapeutical Research Institute
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The possibility of an oral vaccination with deglycosilated chain-A ricin (DGCA)-nanoparticles was investigated. Oral vaccination with DGCA in form of a solution or adsorbed to poly(methyl methacrylate) nanoparticles (PMMA nanoparticles) suspended in an aqueous medium leads to an about 5/ % protection of mice after an aerosol challenge with ricin. All other nanoparticle products, including suspension in a hydrophobic vehicle (paraffin) or the employment of polylactic acid did not achieve a significant protection. Protection did not correlate at all with ELISA antibodies against ricin after oral as well as subcutaneous vaccination.

Peroral absorption studies with ^{125}I-deglycosilated chain-A ricin using autoradiography as well as liquid scintillation counting indicated a slightly higher but short-lived uptake into the residual body after adsorption of the antigen to PMMA nanoparticles suspended in saline.

14. SUBJECT TERMS
Ricin, deglycosilated chain-A ricin (DGCA), vaccination, protection, ELISA antibodies, nanoparticle

15. NUMBER OF PAGES
52

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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4. Table of Contents

5. Introduction

Peroral administration of vaccines is significantly more convenient than parenteral immunization. For this reason, peroral vaccination is much better suited for the mass immunization of soldiers or for administration in the field. In addition, peroral administration may enable the induction of mucosal antibodies and IgA. Peroral vaccines be improved by the employment of adjuvants. These adjuvants can protect the antigen from degradation in the gastro-intestinal tract and may enable the uptake by specialized immuno-competent cells in the intestine, as for instance the M-cells and the Peyer's patches (1-3). Among the most promising adjuvants for peroral administration are nanoparticles. Nanoparticles are definded as colloidal polymer particles (size range 10 nm – 1000 nm) into which drugs or antigens are incorporated or to which these materials are adsorbed or chemically bound (4). These nanoparticles were shown to be absorbed in the gastro-intestinal tract in a certain amount, depending on the particle size (5-8) as well as the composition of the vehicle in which the particles were administered (9). In the present study poly(methyl methacrylate) as well as polylactic acid nanoparticles were developed and investigated for their use as adjuvants for the peroral vaccination with deglycosilated chain-A ricin.

6. Body

6.I. Development of Nanoparticle Adjuvants

6.I.I. Materials

- Deglycosilated chain-A ricin (DGCA) was obtained from Dr. M. Kende, Toxicology Division, USAMRIID
- ^{125} Deglycosilated chain-A ricin, spez. activity: 2,96 MBq/ mmol (Amersham Pharmacia Biotech, Freiburg, Germany)
- Poly(L-lactide was purchased from Boehringer Ingelheim (Germany), i.e. Resomer L206 with an average molecurlar weight 110100.
- Methyl methacrylate, E. Merck, Darmstadt (Germany).
- Polyvinyl alcohol (PVA) with an average molecular weight 13.000 - 23.000 was purchased from Aldrich (Germany).
- Dichloromethane, E. Merck, Darmstadt (Germany)
- Acetone, E. Merck, Darmstadt (Germany)
- Human serum albumin, 96 - 99 % albumin, Sigma (Germany)
- Magnesium chloride hexahydrate, Fluka Chemika (Switzerland)
- Paraffin subliq., E. Merck, Darmstadt, (Germany)
- Phosphate buffered saline (PBS) (pH 7,4)

NaC1	8.0 g
KC1	0.2 g
Na2HP04 x 2 H2O	1.44 g
KH2PO4	0.2 g
Aqua purif. ad	1000.0 g

- Automatic Gamma Counter 1272 (Wallac Distribution GmbH, Freiburg, Germany)
- Jung Cryopolycut (Leica, Bensheim, Germany)
- Branson Bransonic 220

6.1.2 Methods

Preparation of poly(l-lactide) (PLA) nanoparticles coated with polyvinylalcohol

The PLA-nanoparticles were prepared by a high pressure emulsification-solvent evaporation method. A formulation based on a 5 % (w/v) biodegradable polymer organic solution, 0,5 % w/v polyvinylalcohol aqueous solution, and a phase volume ratio 2/10 (organic phase volume/aqueous phase volume) was used. Figure 1 displays a scheme of the basic procedure for the preparation of nanoparticles and basic formulation. One g polylactid acid was dissolved in 20 ml of dichloromethane by sonication. For the preemulsification (Table 1) this organic phase was dispersed in 100 ml of an aqueous PVA solution using a high speed homogenizer (Ulta-Turrax, IKA-Labortechnik, Germany) for 2 min at 8000 rpm. The resulting emulsion was subjected to homogenization in a high-pressure homogenizer (APV-Rannie, Model Mini-Lab, Type 8.304, Denmark) and recycled three times at an operating pressure of 400 bar. The micronized emulsions were stirred magnetically for 24 h at room temperature to evaporate the solvent (dichloromethane). The resulting dispersion was filtered through a sintered glass filter (G3), and pelleted by ultra-centrifugation (100.000 g, 60 min: Beckman L-80, U.S.A.) to eliminate excess PVA and dichloromethane. The nanoparticles were resuspended in distilled water. This purtification procedure was carried out three times. After this process, the suspension was freeze-dried for 24 h (Lyovac GT2, Leybold, Germany).

The mean diameter and particle size distribution of the resulting nanoparticle preparation was measured by photon correlation spectroscopy (PCS) (BI2030AT Goniometer, Brookhaven Instrument Holtsville, U.S.A.) (Table 2). For the studies of the influence of several manufacturing and fomulation parameters on the physical properties of nanoparticles, i. e. evaporation conditions, pH of aqueous phase, solvents, etc. were investigated.

Unless specially mentioned, this basic procedure and formulation were used.

Figure 1: Basic procedure and formulation for preparation of PLA nanoparticles

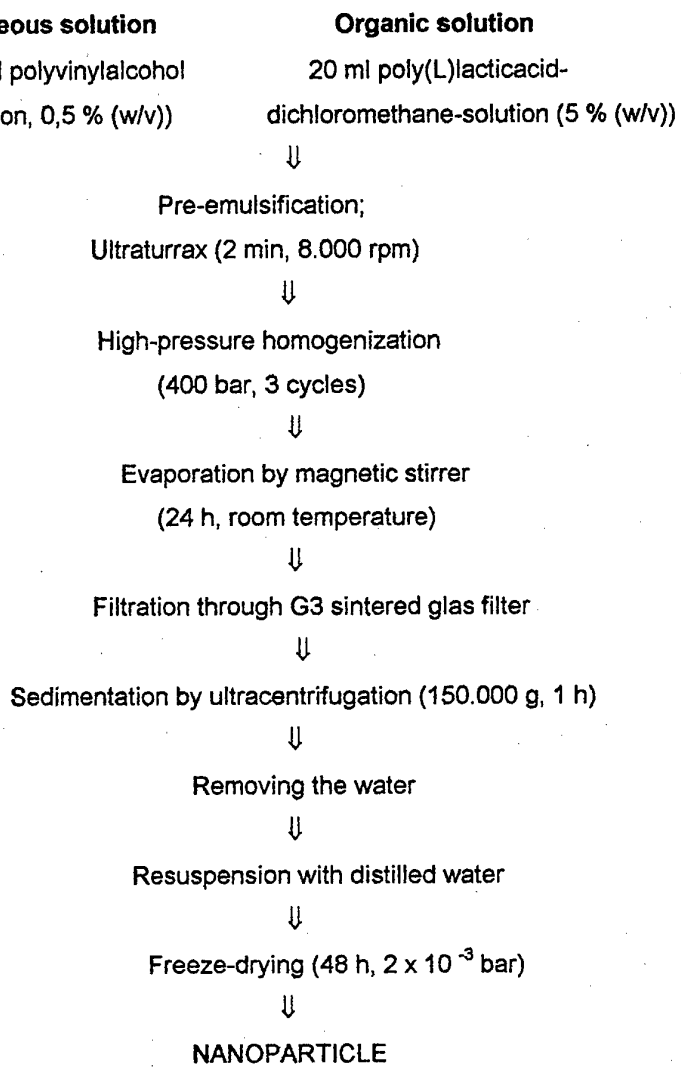


Table 1: Effects of pre-emulsification conditions on nanoparticle size

Pre-emusification conditions		Mean diameter (nm) before HPH		after HPH 3 *		after HPH 5 *
8000 rpm	2 min	1207 ± 114		210.8 ± 2.2		201.4 ± 3.6
13500 rpm	1 min	421 ± 9		213.5 ± 1.5		196.3 ± 3.6

HPH: High-pressure homogenizer
(*) numer of cycles

Various manufacturing conditions and formulation variables influenced the size of nanoparticles as shown in Table 3. For PLA concentrations between 1 and 5 % w/v the nanoparticles possessed a particle size of less than 300 nm.

Preparation of poly(L-lactide) nanoparticles coated with human serum albumin

In addition to its function as a coating agent that is degradable in the gut, albumin acts as an emulsifyer. Therefore it was used as a second coating material for the PLA nanoparticles. Albumin-coated PLA nanoparticles also were obtained by high-pressure-homogenisation and emulsification-solvent evaporation. The diameter of the resulting suspensions was measured using photon correlation spectroscopy (PCS) before lyopilisation and after resuspending in PBS (Table 4).

Table 2: Diameter determination by photon correlation spectroscopy

Minutes	Eff. Diam	Poly.	Eff. Diam.	Poly.	Eff. Diam.	Poly
Sample 1	256.7	0.2788	258.9	0.2793	243.4	0.2322
Sample 2						
10	575.9	0.3875	571.4	0.3842	574.3	0.3866
15	411.4	0.2693	443.6	0.2845	493.3	0.3221
20	383.7	0.2847	395.7	0.2797	389.5	0.2833
60	334.1	0.2226	321.8	0.2449	320.2	0.2231
Sample 3						
10	815.3	3.765	935.2	0.3172	988.15	0.3544
15	769.5	0.2953	815.3	0.3198	853.2	0.2946
20	735.9	0.3256	759.2	0.3863	789.8	0.3153
60	743.8	0.3021	724.9	0.3261	712.9	0.2609

Sample 1: PLA nanoparticle fraction (coated with PVA) before lyophilisation

Sample 2: PLA nanoparticle fraction (coated with PVA) after resuspending by using bath sonocation

Sample 3: PLA nanoparticle fraction (coated with PVA) after resuspending by using probe sonication

Table 3: Effects of manufacturing conditions and formulation on nanoparticle size

PLA conc. (% w/v)	PVA conc. (%w/v)	Phase ratio *	Operating pressure	Number of cycles	Eff. diameter (nm)	S.D.	Polydispers index
1	0.5		200	3	211.9	7.8	0.15
1	0.5		400	5	183.9	6.7	0.15
1	0.5		800	3	163.5	3.5	0.18
1	0.5		400	3	264.5	4.7	0.11
1	0.5		400	3	191.3	4.2	0.17
5	0.5		400	3	259.4	3.8	0.11
1	0.25		400	3	264.1	6.9	0.12
1	2		400	3	173.9	2.1	0.17

Table 4: Eff. diameter of PLA nanoparticles coated with human serum albumin

	1	2	3	Average	S.D.
Eff. diameter	251.9	236.4	228.6	239.0	11.9
Polydisp.	0.2073	0.2872	0.2670	0.2538	0.2538

The influence of the dispersing agents on the size of nanoparticles is shown in Table 5. The human serum albumine (HSA) is an excellent surface active agent. Due to this property both, HSA as well as PVA, enable a reduction in particle size.

Table 5: Effects of dispersing agents on nanoparticle size

Surfactant	concentration	1	2	3	Polydisp. Index
PVA	0.5	222.1	218.1	210.4	0.15
PVA	2	180.6	187.5	189.6	0.17
PVA	5	171.0	180.4	185.5	0.19
HSA	0.5	261.9	260.2	266.9	0.25
HSA	1	202.9	190.1	197.0	0.25

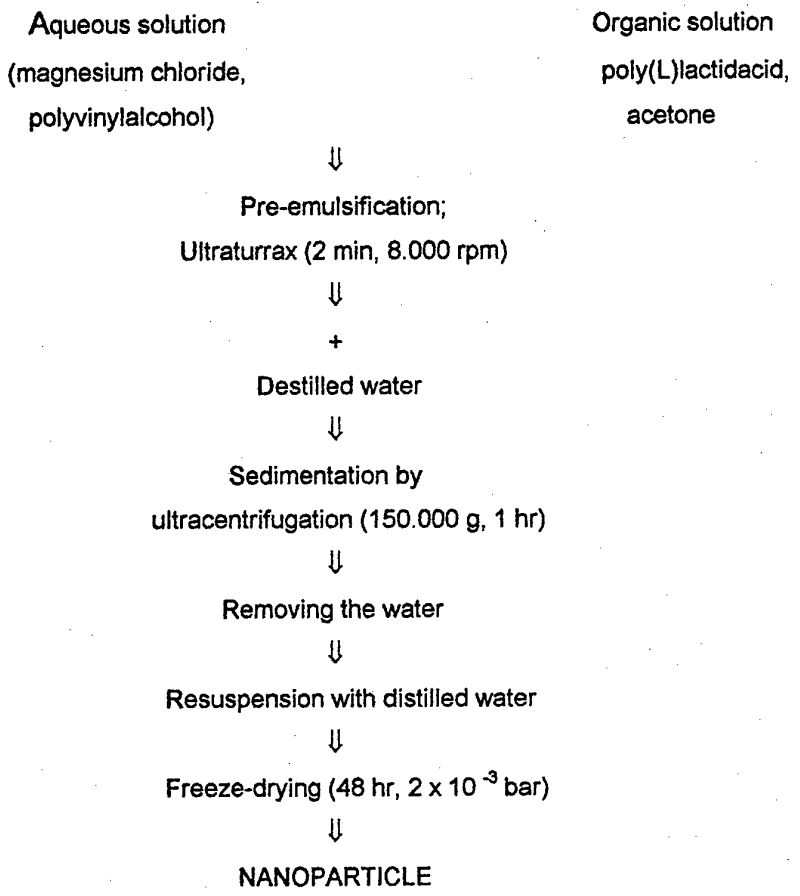
Preparation of poly(L-lactide) nanoparticles coated with polyvinyl-alcohol or human serum albumin by the method of Ibrahim et al.

Because of the destroying effect of dichloromethane to ricin the method to produce poly(L-lactide) nanoparticles was changed. Instead of dichloro-methane acetone was used and the particles were prepared employing the method of Ibrahim et al.

A formulation based on 18 % (w/v) biodegradable polymer in acetone and a 3.7 % (w/v) polyvinylalcohol / 60,5 % (w/v) magnesiumchloride containing aqueous solution was used at a phase volume ratio of 2/3 (organic phase volume/aqueous phase volume).

Figure 2 displays a scheme of the basic procedure for the preparation of PLA nanoparticles and basic formulation employing the method of Ibrahim et al. Specificly, 4.04 g polylactid acid was dissolved in 22.4 ml of acetone by sonication.This organic phase was dispersed in 33.6 ml water containing PVA (3.4 g) and magnesium chloride (20.2 g) using a high speed homo-genizer (Ultra-Turrax, IKA-Labortechnik, Germany) for 2 min at 8000 rpm. 33.6 ml destilled water was added to the resulting emulsion that was then subjected to high-pressure homogenization (APV-Rannie, Model Mini-Lab,

Figure 2: Basic procedure for the preparation of PLA nanoparticles employing the method of Ibrahim et al.



Type 8.304, Denmark). The emulsion was recycled three times at an operating pressure of 400 bar. The micronized emulsions was washed four times with destilled water using ultracentrifugation (18000 rpm, 30 min; Beckman L-80, U.S.A.) to eliminate the acetone. The resulting nanoparticles were than resuspended in 60 ml distilled water. After this process, the suspension was freeze-dried for 24 h (Lyovac GT2, Leybold, Germany).

Table 6: Diameter determination by using photon correlation spectroscopy

	Eff.Diam.	Poly.	Eff. Diam.	Poly.	Eff. Diam.	Poly.Ind.
Sample 1	850.9	0.3944	975.8	0.3155	994.3	0.3995
Sample 2	796.5	0.2985	885.9	0.2645	943.9	0.2486
Sample 3	956.3	0.2654	810.9	0.2596	923.9	0.3765
Sample 4	756.9	0.2385	780.9	0.2465	750.9	0.2568

Sample 1: Without the high-pressure homogenization
Sample 2: High-pressure homogenizer (three cycles)
Sample 3: High-pressure homogenizer (four cycles)
Sample 4: High-pressure homogenizer (five cycles)

Table 7: Uncoated PMMA with adsorbed DGCA in different suspension mediums and two control groups

Group	Particle	Vaccine	Suspension-medium	Mice/Group
1	PMMA	DC GA adsorbed	PBS (pH 7.4)	10
2	PMMA	DC GA adsorbed	Miglyol	10
3	PMMA	DC GA adsorbed	Paraffine	10
4	PMMA	DC GA adsorbed	Olive oil	10
5	PMMA	DC GA adsorbed	Polyethylene glycole	10
6	None	DGCA aqueous	none	10

A dose of 25 ug vaccine and a volume of 0.5 ml/inj. was administered orally on day 1, 2, 3, and 14 by gavage. The mice were bled by the tail vein to collect 0.3 ml blood at day 28 for determination of antiricin IgG by ELISA.

Preparation of poly(methyl methacrylate) (PMMA) nanoparticles with incorporated DGCA

As a first step the methyl methacrylate which is stabilised with 100 ppm hydrochinone was purified from this polymerization inhibitor by extraction with a solution of sodium hydroxide (5 % w/v) and sodium chloride (20 % w/v). This procedure was repeated three times. After this the methyl methacrylate was washed three times with destilled water.

Preparation 1	Preparation 2	Preparation 3
2.5 mg DGCA	2.5 mg DGCA	2.5 mg DGCA
20 ml dest. water	20 ml dest. water	20 ml dest. water
0.2 g monomer (MMA)	0.2 g monomer (MMA)	-

The nanoparticles in preparation 1 and 2 were produced by polymerization using ^{60}Co-y-irradiation at a dose of 150 krad (=1,5 kGy) and a dose rate of 1 krad/min (=0,602 kGy/h). After this preparation 2 was lyophilized for 24 h (Lyovac GT2, Leybold, Germany) and resuspended in paraffin.
Preparation 3 was not irradiated.

Table 8: ELISA determination of antiricin IgG

Groups (see Table 7)							Average	Geometric Means
1	12900	502	7590	417			5352.25	2127.74
2	3020	709	269	24600			7149.5	1940.15
3	13200	25700	20400	6310	40960		21314.0	170803.50
4	159	10300	398				3619.0	867.04
5	1150	537					843.5	785.84
6	7760	32400	954	7760	2190	26900	12994.0	6918.35
LOGS								
1	4,11	2.7	3.88	2.62			3.33	
2	3.48	2.85	2.43	4.39			3.29	
3	4.12	4.41	4.31	3.8	4.61		4.25	
4	2.2	4.01	2.6				2.94	
5	3.06	2.73					2.90	
6	3.89	4.51	2.98	3.89	3.34	4.43	3.84	

The determination of antiricin IgG in mice after vaccination with the different above listed vaccine preparations shows that DGCA absorbed on PMMA suspended in paraffin yielded the highest ELISA antibody response. This response was signifiantly higher than that of sample 6 (aqueous DGCA). Because of this result in the following determinations nanoparticles with DGCA were produced using paraffin as the suspension medium.

Preparation of poly(methyl methacrylate) (PMMA) nanoparticles with adsorbed DGCA

PMMA-nanoparticles were prepared by a method of Riddle (10), Tessmar (11). A solution of 1.0 % methyl methacrylate in destilled water was polymerized with a dose of 500 krad and a dose rate of 2,2 krad/min by using a ^{60}Co-gamma-irradiation (12). The resulting particle-preparation was freeze-dried and stored at room temperature. The resuspension procedure of the nanoparticle preparation was optimized prior to adsorption to ensure effective adsorption of the antitoxin.
The optimal conditions for resuspending the nanoparticle preparation are as follows: The lyophilized particles are resuspended phosphate buffered saline solution (PBS) in a concentration of a 2 % (w/v) suspension by using sonication (Branson Bransonic 220) and an operating temperature of 4 °C for two hours per day; this procedure was repeated on three following days. Afterwards, the basic suspension of PMMA nanoparticles was diluted with PBS to obtain a 0.5 % suspension; the dilution medium contained the inactived chain-A ricin (DGCA). The suspension was stirred for 24 h at temperature of 4 °C to adsorb the antitoxin. After this process, the resulting suspension was freeze-dried for 24 h (Lyovac GT2, Leybold, Germany).

Equal parts of the preparation were resuspended in the different delivery mediums for the oral vaccine: olive oil, polyethylenglycole, paraffin, miglyol and phosphate buffered saline.

6.2. Testing of the Optimized Vaccine Preparations

The vaccine preparations were tested by USAMRIID in Fort Detrick, Dr. Kende, Immunomodulators Section, Dept. of Antiviral Studies, Virology Dir.

The different nanoparticle preparations made of PMMA, PLA, or PLGA were suspended in water or paraffin to investigate the vehicle hydrophobicity influence, since previous body distribution using similar ^{14}C-labelled PMMA nanoparticles showed that the vehicle has an important influence on particle uptake and body distribution (9).
The most relevant results of these experiments are summerized in Table 9.

These results show two important findings: The first finding is that only PMMA in aqueous suspension and the DGCA aqueous vaccine without additives administered orally or also the subcutaneous vaccines yielded significant protection. The second, even more important finding shows that ELISA antibodies and protection do not correlate at all. In only one case, aqueous DGCA without adjuvant administered subcutaneously, high ELISA antibody titers as well as a good protection (4 out of 5 animals) was obtained. In many other cases rather a negative correlation was observed, i.e. high ELISA antibody titers and a low protection as with PMMA in paraffin, PLA in paraffin, PLGA in paraffin, administered orally, and aqueous PLA administered subcutaneously or, vice versa, low ELISA titers and a rather good protection with the aqueous PMMA or the DGCA aqueous vaccine. In order to shed more light into these rather puzzling results, body distribution experiments were performed with the aqueous DGCA vaccine without additives and the PMMA nanoparticle uspended either in saline or in parafine, using ^{125}I-labled deglycosylated chain-A ricin. The ricin dose was the same as in the preceeding immunization experiments.

Table 9

Geometric mean titer determined by ELISA and protection against lethal aerosol challenge with ricin

Experimental group	Geometric mean titer	Surviving mice on day 10 Survivers / total number
Oral Vaccination		
Aqueous PMMA	2667	2/4
PMMA in paraffin	12303	0/7
Aqueous PLA	780	0/9
PLA in paraffin	24044	0/8
Aqueous PLGA	1387	0/10
PLGA in paraffin	7998	0/8
DGCA (aqueous vaccine)4/50		5/8
Subcutaneous injection		
Aqueous PMMA	89743	4/5
Aqueous PLA	124451	0/5
Aqueous PLGA	54325	5/5
DGCA (aqueous vaccine)	163117	4/5
Control	-	0/6

6.3. Body Distribution Studies with ^{125}I-Deglycosylated Chain-A Ricin (^{125}I-DGCA) in Mice

6.3.1 Preparation of poly(methyl methacrylate) (PMMA) nanoparticles with absorbed deglycosilated chain-A ricin (DGCA)

PMMA-nanoparticles were prepared by a method of Riddle (10), Tessmar (11) as described above under 6.1.2.: A solution of 0,1 % methyl methacrylate in destilled water was polymerized with a dose of 5 kGy and a dose rate of 22 kGy/min by using 60 C°-gamma irradiation (12). The resuspension of the nanoparticle preparation was optimized for the adsorption of the antitoxin. The optimal condintions for resuspending the nanoparticle preparation are as follows: The lyophilized particles are resuspended in PBS in a concentration of 2 % (w/v) suspension by using sonication (Branson Bransonic 220) at 4°C for two hours per day and this repeated on three following days. The suspension of PMMA-nanoparticles was diluted to obtain a 0.5% suspension with PBS, the dilution medium contained the inactivated antitoxin ricin (chain-A). The suspension was stirred for 24 h at 4 °C to adsorb the antitoxin. The resulting suspension was freeze-dried (Lyovac GT2, Leybold, Germany) and equal parts of the preparation were suspended in PBS or paraffin for the oral vaccine.

6.3.2. Whole body autoradiography study

The whole body autoradiography technique permits a general view of the distribution pattern of the ^{125}I-ricin chain-A in practically all tissues of the body.

Animal experiments

The animals were kept under standard conditions with free access to water and food. The nanoparticle preparations and the ricin solution were administered orally by gavage. For each preparation and each sampling time

point (1 and 4 hours) one mouse (female CD 1, Harlan Winkelmann, Borchen, Germany) of a body weight of about 30 g was treated by a single oral dose of 25 µg ^{125}I - DGCA in a volume of 0.2 ml or one of the following three preparations: aqueous preparation of DGCA without nanoparticles, DGCA adsorbed to PMMA nanoparticles in saline, or DGCA adsorbed to PMMA nanoparticles in paraffin. After dosing the animals were kept in individual cages until they were sacrificed after 1 or 4 hours using CO_2.

Autoradiography

The sacrificed animals were mounted into a microtome stage as follows: As the mounting medium a semiliquid gel of caroboxymethyl cellulose was used. During the mounting and the consecutive freezing, the stage was surrounded by a metal frame. The bottom of the box thus formed was covered with the mounting medium, and the mouse was placed onto this layer. Additional mounting medium was then filled into the box until the animal was covered. The whole box then was lowered slowly into the freezing liquid container. Freezing time was about 20 min.
Before sectioning begins, the frozen block was allowed to reach the temperature of the cryostat (-20 ^{0}C). The frozen animal was sectioned sagitally until a section surface of interest appeared. Before a final section was taken, a piece of transparent tape was fastend onto the section surface of the residual block. The microtome knife then cut under the tape, and the sliced section remained attached to the tape. The resulting section thickness was 25 um. The sections were dried by being stored at - 20 $^{\circ}$ C in the cryosat. After that, the slices were placed for a week on a radio sensor plate i.e. an imaging plate (IP) obtained from Fuji Photo Film Co Ltd. After the exposure time, the slices were scanned by a computer.

Result:

The main radioactivity remains in the gastro-intestinal tract. Negligible levels of radioactivity, however, were distributed to the residual body. The amounts were specially high with PMMA in saline, visually higher than with the other preparations.

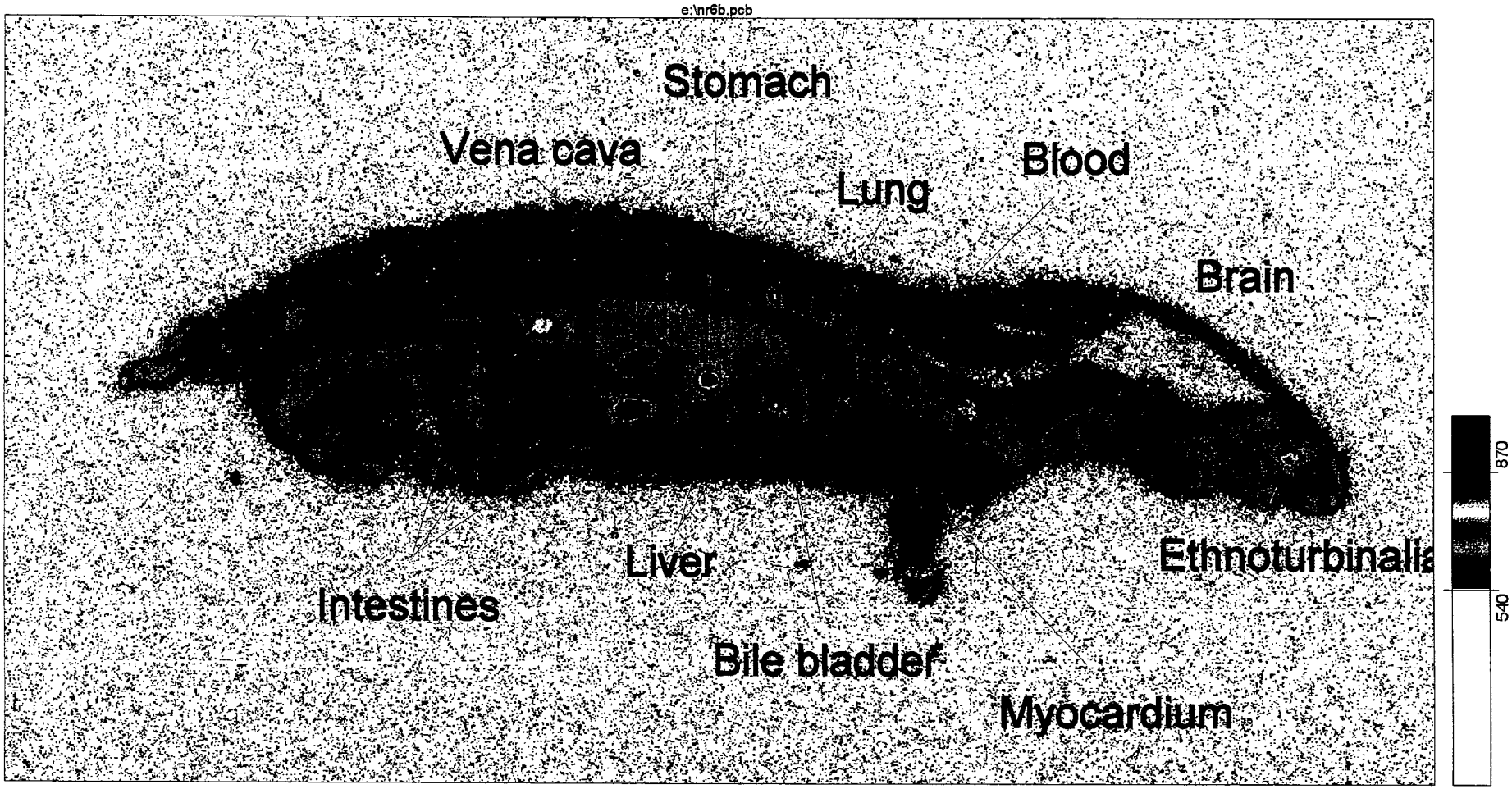


Fig. 3: Autoradiography of a mouse 1 hour after oral administration of ricin chain-A (25μg) solution.

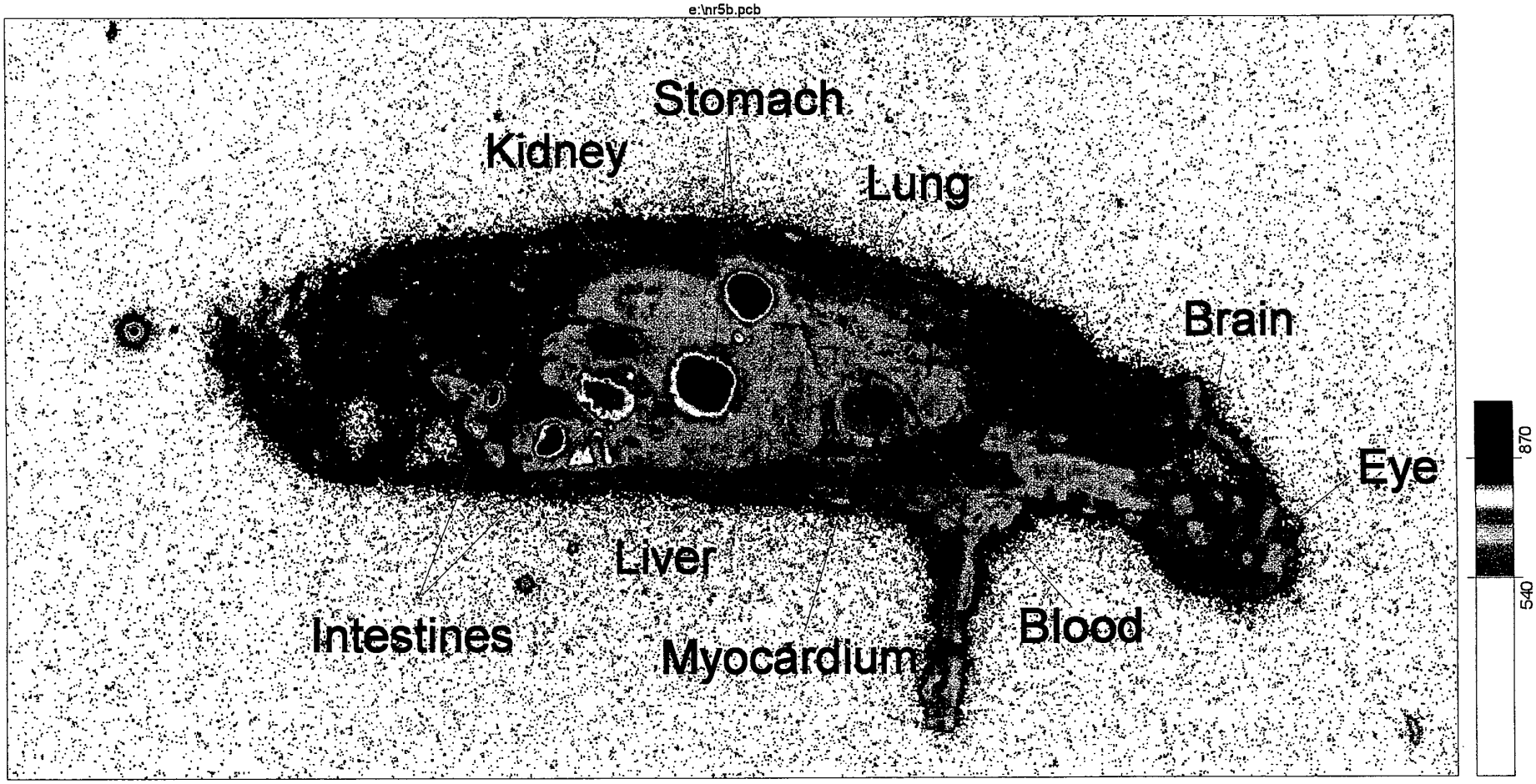


Fig. 4: Autoradiography of a mouse 1 hour after oral administration of ricin chain-A (25µg) adsorbed to PMMA nanoparticles suspended in saline.

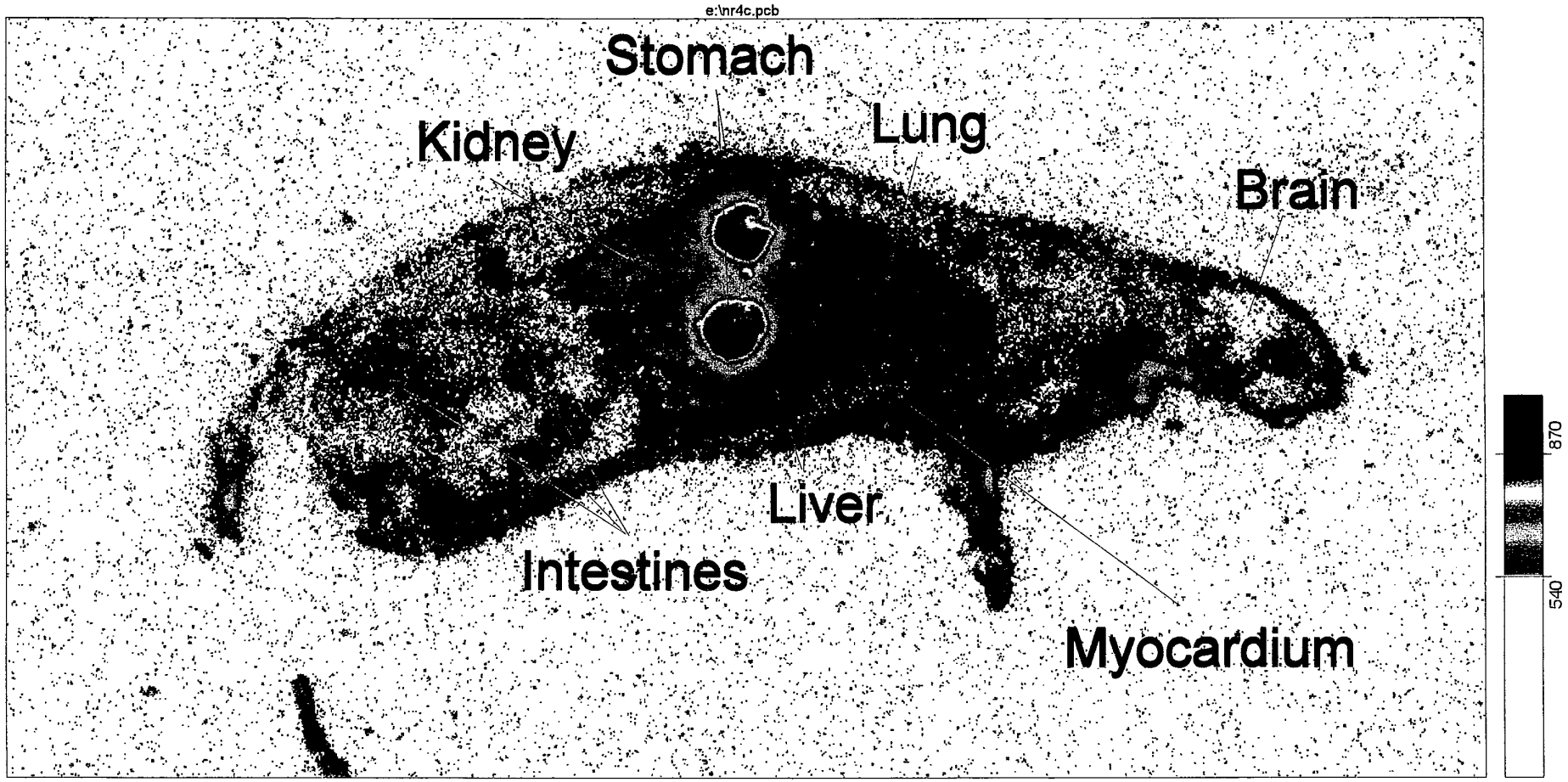


Fig. 5: Autoradiography of a mouse 1 hour after oral administration of ricin chain-A (25μg) adsorbed to PMMA nanoparticles suspended in paraffin.

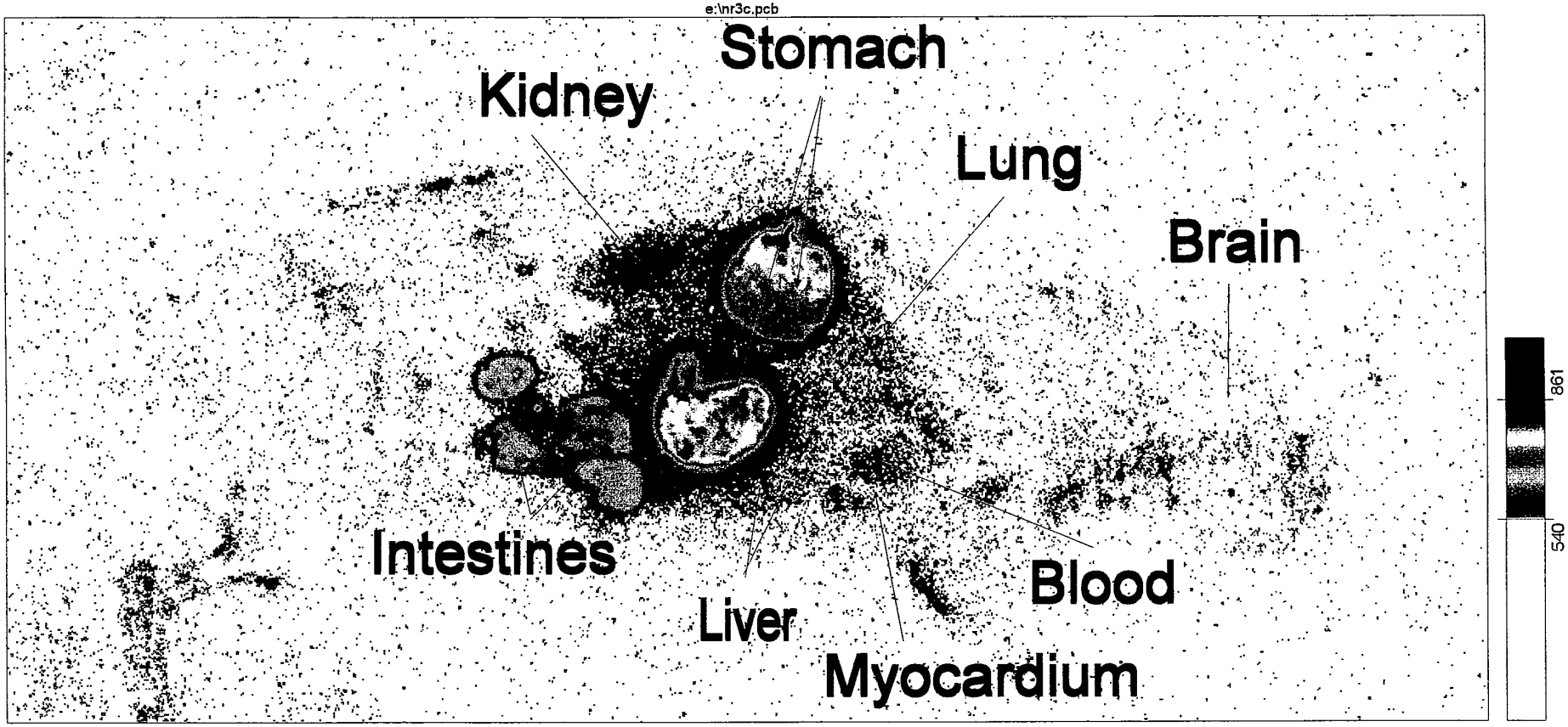


Fig. 6: Autoradiography of a mouse 4 hours after oral administration of ricin chain-A (25μg) solution.

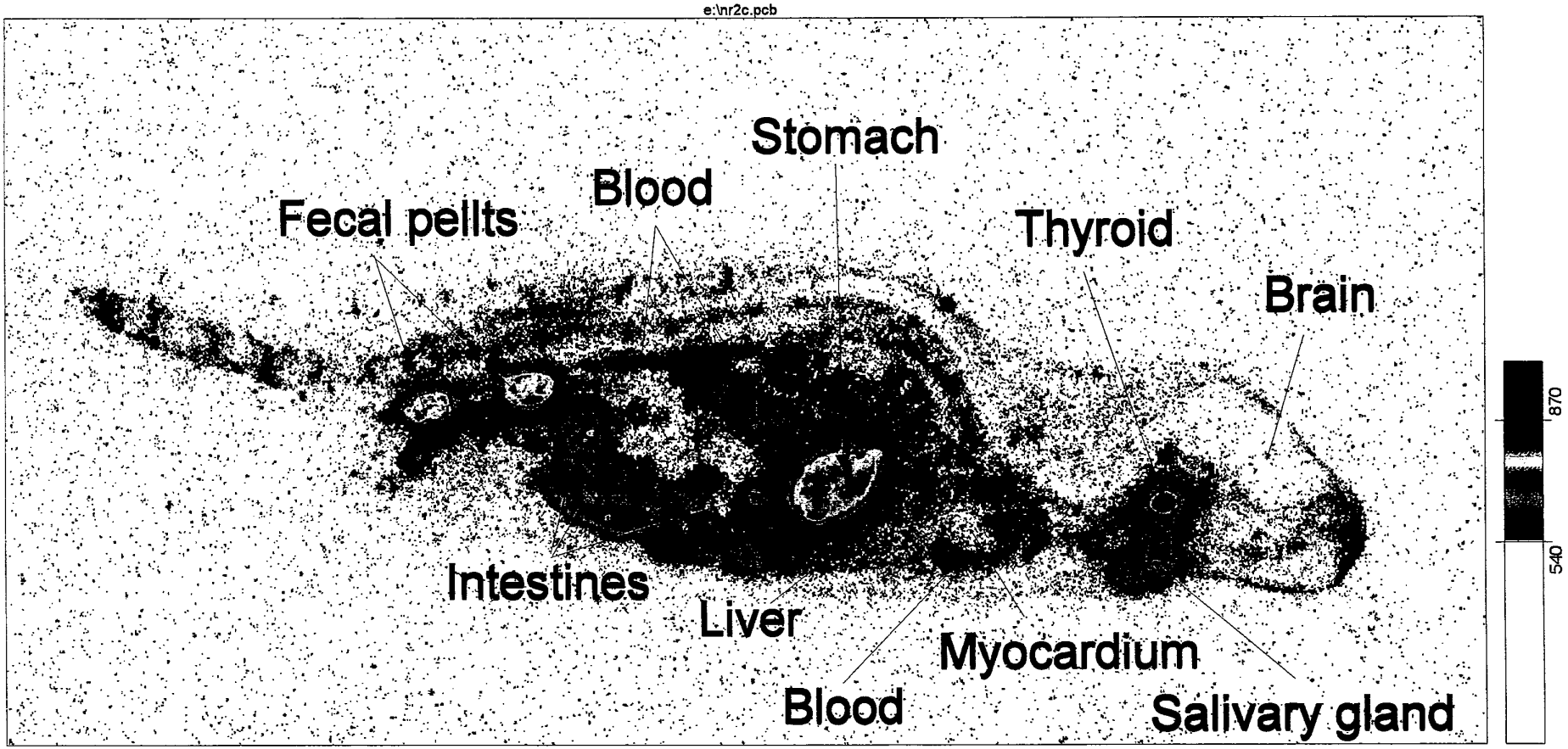


Fig. 7: Autoradiography of a mouse 4 hours after oral administration of ricin chain-A (25μg) adsorbed to PMMA nanoparticles suspended in saline.

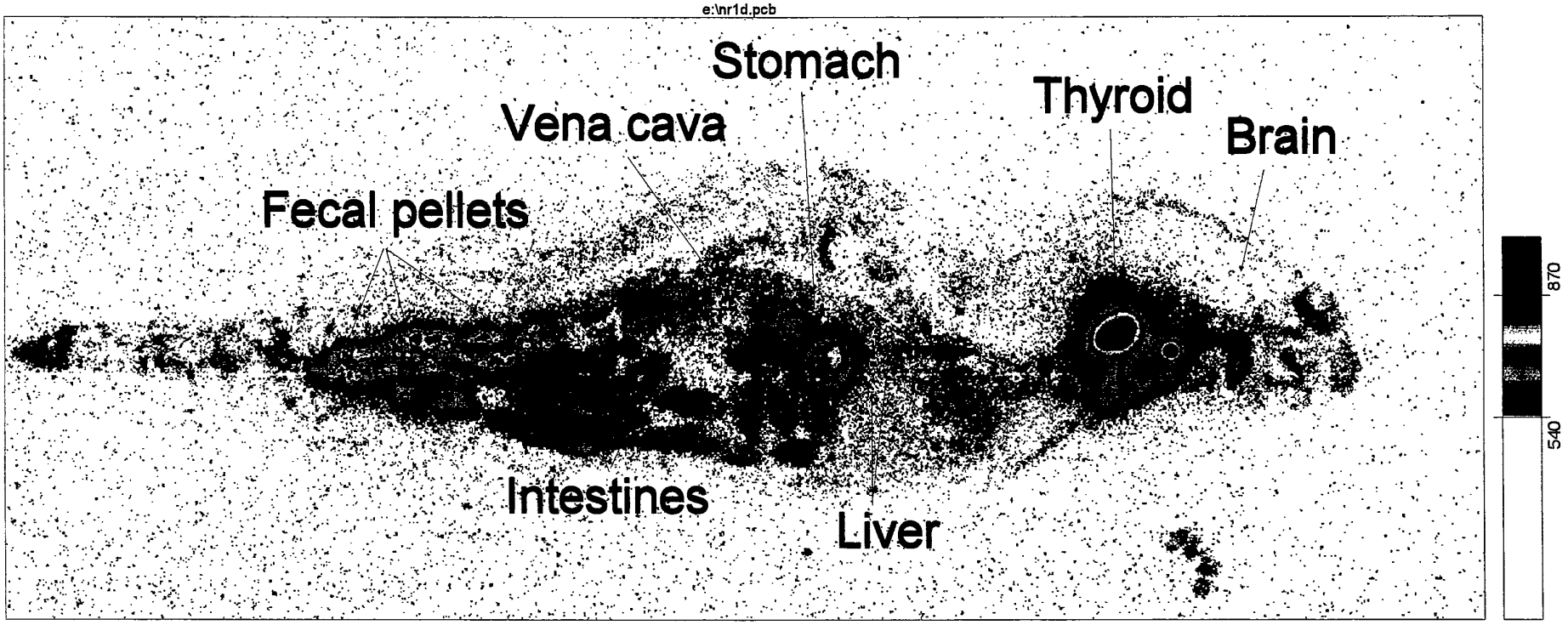


Fig. 8: Autoradiography of a mouse 4 hours after oral administration of ricin chain-A (25µg) adsorbed to PMMA nanoparticles suspended in paraffin.

6.3.3. Body distribution of ^{125}I-deglycosilated chain-A ricin (DGCA) vaccines after oral administration using gamma counting

Animal experiments

The animals were kept under standard conditions with free access to water and food. The nanoparticle preparations and the ricin solution were administered by oral gavage. For each preparation and each sampling time point, four mice (CD 1, Harlan Winkelmann, Borchen, Germany) of a body weight of about 30 g were treated with a single dose of 25 µg ricin and a volume of 0.2 ml. in form of one of the following three preparations:
Aqueous ^{125}I-DGCA,
^{125}I-DGCA adsorbed to PMMA nanoparticles in saline,
^{125}I-DGCA adsorbed to PMMA nanoparticles in paraffin.

After administration of the ^{125}I-labelled ricin A-chain solution or the nanoparticle preparations the animals were sacrificed at 15, 30 min, 1, 2, 4, 8 hours, 1, 2, 4 days, and 1, 2, 4 weeks by placing in CO_2.

Determination of ^{125}I-radioactivity

The animals were dissected and each organ was removed and two samples of each organ or tissue accurately weighed into a scintillation vial.
The samples were counted in a gamma counter (LKB-Wallac, Clini-Gamma, Wallac Oy, Turku, Finnland). The weight of the blood, bone, marrow, muscles were estimated from the total body weight and employing the following percentages: bone marrow 1,3%, muscles 45 % and blood 7%.

Table 10

Percentage of the ^{125}I-deglycosilated chain-A ricin in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil in various organs and tissues after oral adminitration

Time : 15 min

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,17	0,08	0,25	0,09	0,43	0,16
lungs	0,59	0,29	0,96	0,42	1,04	0,28
liver	0,73	0,24	1,14	0,46	1,57	0,46
spleen	0,12	0,05	0,19	0,08	0,28	0,04
kidney	0,92	0,29	1,32	0,54	1,71	0,57
muscles	2,42	0,82	3,38	1,57	2,39	1,83
blood	5,31	1,56	7,18	3,16	8,12	2,45
bone marrow	0,78	0,23	0,92	0,58	0,88	0,31
Stomach	4,61	0,87	8,67	2,93	4,65	1,56
Stomach content	35,01	9,69	30,43	11,09	12,87	4,77
Small intestine	1,25	1,22	2,01	1,63	3,09	2,79
S. intestine content	2,07	1,54	4,29	2,70	10,01	5,22
Colon	0,33	0,32	0,83	0,83	0,68	0,58
Colon content	0,21	0,24	0,34	0,35	0,18	0,19

Time : 30 min

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,28	0,11	0,39	0,08	0,18	0,10
lungs	0,78	0,25	1,19	0,41	0,64	0,35
liver	1,21	0,43	1,95	1,00	1,00	0,58
spleen	0,54	0,59	0,46	0,29	0,23	0,15
kidney	1,37	0,29	1,60	0,41	1,00	0,54
muscles	3,48	1,00	4,16	1,19	2,35	1,43
blood	6,47	2,30	10,40	3,52	6,91	4,53
bone marrow	1,12	0,33	2,03	0,90	0,96	0,68
Stomach	5,04	1,37	7,63	0,86	3,09	1,58
Stomach content	26,54	5,27	44,02	11,83	9,11	7,65
Small intestine	1,41	0,41	1,98	1,22	1,19	0,99
S. intestine content	4,17	1,50	4,36	2,00	5,78	3,88
Colon	0,80	0,87	0,76	0,73	0,44	0,54
Colon content	0,28	0,29	0,32	0,31	0,21	0,25

Time : 1 hour

% Dose	Control	± SD	PMMA in saline	± SD	PMMA in paraffin	± SD
heart	0,31	0,05	0,79	0,75	0,22	0,20
lungs	1,41	0,24	0,76	0,24	0,58	0,49
liver	1,90	0,76	1,70	0,53	0,97	0,79
spleen	0,36	0,05	0,41	0,13	0,17	0,10
kidney	1,49	0,09	1,78	0,46	0,86	0,64
muscles	4,98	2,94	4,00	0,96	2,13	1,55
blood	9,34	0,53	10,42	2,79	5,63	4,72
bone marrow	1,52	0,15	1,78	0,55	0,84	0,85
Stomach	7,26	0,80	6,16	2,10	3,89	1,87
Stomach content	13,73	9,16	29,23	9,59	11,28	8,24
Small intestine	1,70	0,82	2,09	0,82	1,47	1,11
S. intestine content	3,01	1,11	5,20	0,72	4,37	2,21
Colon	0,81	0,77	0,67	0,56	0,47	0,58
Colon content	1,13	0,51	0,68	0,77	0,20	0,26

Time : 2 hour

% Dose	Control	± SD	PMMA in saline	± SD	PMMA in paraffin	± SD
heart	0,22	0,08	0,41	0,08	0,25	0,04
lungs	0,79	0,31	1,35	0,44	0,90	0,36
liver	0,96	0,36	1,78	0,45	1,14	0,25
spleen	0,24	0,11	0,32	0,08	0,21	0,09
kidney	1,06	0,35	1,58	0,18	1,19	0,20
muscles	2,52	0,92	4,26	0,43	2,95	0,72
blood	6,48	1,70	11,24	0,27	8,10	1,59
bone marrow	1,15	0,43	1,97	0,22	1,28	0,17
Stomach	5,59	2,48	7,19	2,13	6,62	1,67
Stomach content	31,83	9,50	9,72	2,94	19,49	9,98
Small intestine	1,43	0,61	1,66	0,44	2,39	1,77
S. intestine content	2,01	0,90	2,09	0,49	7,48	4,10
Colon	0,76	0,76	1,19	1,20	0,91	0,80
Colon content	1,50	1,67	2,04	2,45	2,81	3,44

Time : 4 hour

% Dose	Control	± SD	PMMA in saline	± SD	PMMA in paraffin	± SD
heart	0,25	0,22	0,14	0,05	0,41	0,26
lungs	0,58	0,40	0,35	0,09	2,42	1,90
liver	0,72	0,51	0,60	0,17	1,62	1,10
spleen	0,16	0,12	0,11	0,04	0,28	0,20
kidney	0,79	0,48	0,58	0,11	1,40	1,05
muscles	2,15	1,45	1,43	0,50	3,92	2,76
blood	5,65	4,06	3,45	0,89	10,06	8,10
bone marrow	0,86	0,66	0,58	0,21	2,31	2,34
Stomach	2,48	1,74	1,77	0,45	4,52	3,55
Stomach content	6,92	4,73	13,13	9,66	13,38	6,09
Small intestine	0,86	0,54	0,81	0,29	1,35	1,26
S. intestine content	1,21	0,43	1,61	0,27	3,51	2,17
Colon	0,60	0,42	0,35	0,33	0,95	0,96
Colon content	3,08	3,13	4,31	3,91	5,86	4,54

Time :8 hour

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,25	0,12	0,22	0,05	0,06	0,03
lungs	0,72	0,30	0,63	0,21	0,17	0,06
liver	1,11	0,53	1,03	0,29	0,26	0,07
spleen	0,25	0,12	0,20	0,05	0,04	0,01
kidney	0,98	0,37	0,91	0,23	0,25	0,08
muscles	2,12	0,94	2,35	0,60	0,61	0,21
blood	7,03	3,34	6,56	1,60	1,36	0,37
bone marrow	0,79	0,34	1,10	0,49	0,20	0,05
Stomach	3,73	2,07	2,89	0,66	0,84	0,73
Stomach content	4,97	2,74	6,55	3,08	9,25	9,13
Small intestine	1,07	0,58	0,94	0,47	0,31	0,19
S. intestine content	2,01	0,89	1,72	1,22	0,79	0,23
Colon	0,65	0,59	0,60	0,54	0,18	0,16
Colon content	4,83	4,83	4,44	3,64	1,33	1,28

Time : 1 day

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,01	0,01	0,00	0,01	0,01	0,02
lungs	0,02	0,01	0,01	0,01	0,03	0,04
liver	0,04	0,01	0,03	0,02	0,06	0,07
spleen	0,00	0,00	0,00	0,00	0,01	0,01
kidney	0,02	0,01	0,01	0,01	0,05	0,07
muscles	0,00	0,00	0,00	0,00	0,06	0,11
blood	0,12	0,05	0,05	0,04	0,31	0,42
bone marrow	0,00	0,01	0,00	0,00	0,02	0,03
Stomach	0,07	0,03	0,04	0,02	0,43	0,66
Stomach content	0,13	0,05	0,06	0,07	3,34	5,72
Small intestine	0,03	0,02	0,02	0,01	0,08	0,10
S. intestine content	0,10	0,05	0,03	0,03	0,33	0,53
Colon	0,03	0,03	0,01	0,02	0,05	0,06
Colon content	0,24	0,22	0,10	0,11	0,51	0,62

Time : 2 days

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,00	0,00	0,00	0,01	0,01	0,02
lungs	0,02	0,01	0,07	0,12	0,00	0,01
liver	0,01	0,01	0,02	0,01	0,01	0,00
spleen	0,00	0,00	0,00	0,00	0,00	0,00
kidney	0,00	0,00	0,00	0,00	0,00	0,00
muscles	0,06	0,04	0,00	0,00	0,00	0,00
blood	0,01	0,02	0,03	0,01	0,00	0,00
bone marrow	0,20	0,11	0,00	0,00	0,00	0,00
Stomach	0,03	0,01	0,02	0,01	0,01	0,01
Stomach content	0,01	0,01	0,02	0,01	0,04	0,03
Small intestine	0,01	0,01	0,01	0,01	0,01	0,01
S. intestine content	0,01	0,01	0,01	0,01	0,01	0,01
Colon	0,03	0,03	0,01	0,01	0,01	0,01
Colon content	0,00	0,00	0,04	0,04	0,03	0,03

Time : 4 days

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,00	0,00	0,00	0,01	0,01	0,02
lungs	0,00	0,00	0,00	0,00	0,01	0,01
liver	0,00	0,00	0,01	0,00	0,01	0,01
spleen	0,00	0,00	0,00	0,00	0,00	0,00
kidney	0,00	0,00	0,00	0,00	0,00	0,01
muscles	0,00	0,00	0,00	0,00	0,00	0,00
blood	0,00	0,00	0,01	0,01	0,04	0,06
bone marrow	0,00	0,00	0,00	0,00	0,04	0,08
Stomach	0,00	0,00	0,00	0,00	0,01	0,02
Stomach content	0,01	0,00	0,00	0,00	0,03	0,04
Small intestine	0,00	0,01	0,00	0,01	0,01	0,02
S. intestine content	0,01	0,01	0,01	0,01	0,03	0,04
Colon	0,01	0,01	0,01	0,01	0,01	0,01
Colon content	0,02	0,03	0,02	0,02	0,04	0,05

Time : 1 week

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,00	0,00	0,00	0,00	0,00	0,01
lungs	0,00	0,00	0,00	0,00	0,00	0,00
liver	0,00	0,00	0,00	0,00	0,00	0,00
spleen	0,00	0,00	0,00	0,00	0,00	0,00
kidney	0,00	0,00	0,00	0,00	0,00	0,00
muscles	0,00	0,00	0,00	0,00	0,00	0,00
blood	0,00	0,00	0,00	0,00	0,00	0,00
bone marrow	0,00	0,00	0,00	0,00	0,00	0,00
Stomach	0,00	0,00	0,00	0,00	0,00	0,00
Stomach content	0,00	0,00	0,00	0,00	0,00	0,00
Small intestine	0,00	0,00	0,00	0,00	0,00	0,00
S. intestine content	0,00	0,01	0,00	0,00	0,01	0,01
Colon	0,01	0,01	0,00	0,00	0,01	0,01
Colon content	0,01	0,02	0,00	0,00	0,02	0,02

Time :2 weeks

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,00	0,00	0,00	0,00	0,00	0,00
lungs	0,00	0,00	0,00	0,00	0,00	0,00
liver	0,00	0,00	0,00	0,00	0,00	0,00
spleen	0,00	0,00	0,00	0,00	0,00	0,00
kidney	0,00	0,00	0,00	0,00	0,00	0,00
muscles	0,00	0,00	0,00	0,00	0,00	0,00
blood	0,00	0,00	0,00	0,00	0,00	0,00
bone marrow	0,00	0,00	0,00	0,00	0,04	0,06
Stomach	0,00	0,00	0,00	0,00	0,00	0,01
Stomach content	0,00	0,00	0,00	0,00	0,00	0,01
Small intestine	0,00	0,01	0,00	0,00	0,00	0,01
S. intestine content	0,00	0,01	0,00	0,01	0,01	0,01
Colon	0,01	0,01	0,01	0,01	0,01	0,01
Colon content	0,01	0,02	0,01	0,01	0,01	0,02

Time : 4 weeks

% Dose	Control	± SD	PMMA	± SD	PMMA	± SD
			in saline		in paraffin	
heart	0,00	0,01	0,00	0,00	0,00	0,00
lungs	0,01	0,01	0,00	0,00	0,00	0,00
liver	0,00	0,00	0,00	0,00	0,00	0,00
spleen	0,00	0,00	0,00	0,00	0,00	0,00
kidney	0,00	0,00	0,00	0,00	0,00	0,00
muscles	0,00	0,00	0,00	0,00	0,00	0,00
blood	0,00	0,00	0,00	0,00	0,00	0,00
bone marrow	0,00	0,00	0,00	0,00	0,04	0,06
Stomach	0,00	0,00	0,00	0,00	0,00	0,01
Stomach content	0,00	0,00	0,00	0,00	0,00	0,01
Small intestine	0,00	0,00	0,00	0,00	0,00	0,01
S. intestine content	0,00	0,00	0,00	0,00	0,01	0,01
Colon	0,00	0,00	0,00	0,00	0,01	0,01
Colon content	0,00	0,00	0,00	0,00	0,01	0,02

Fig. 9: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in the stomach content after oral administration.

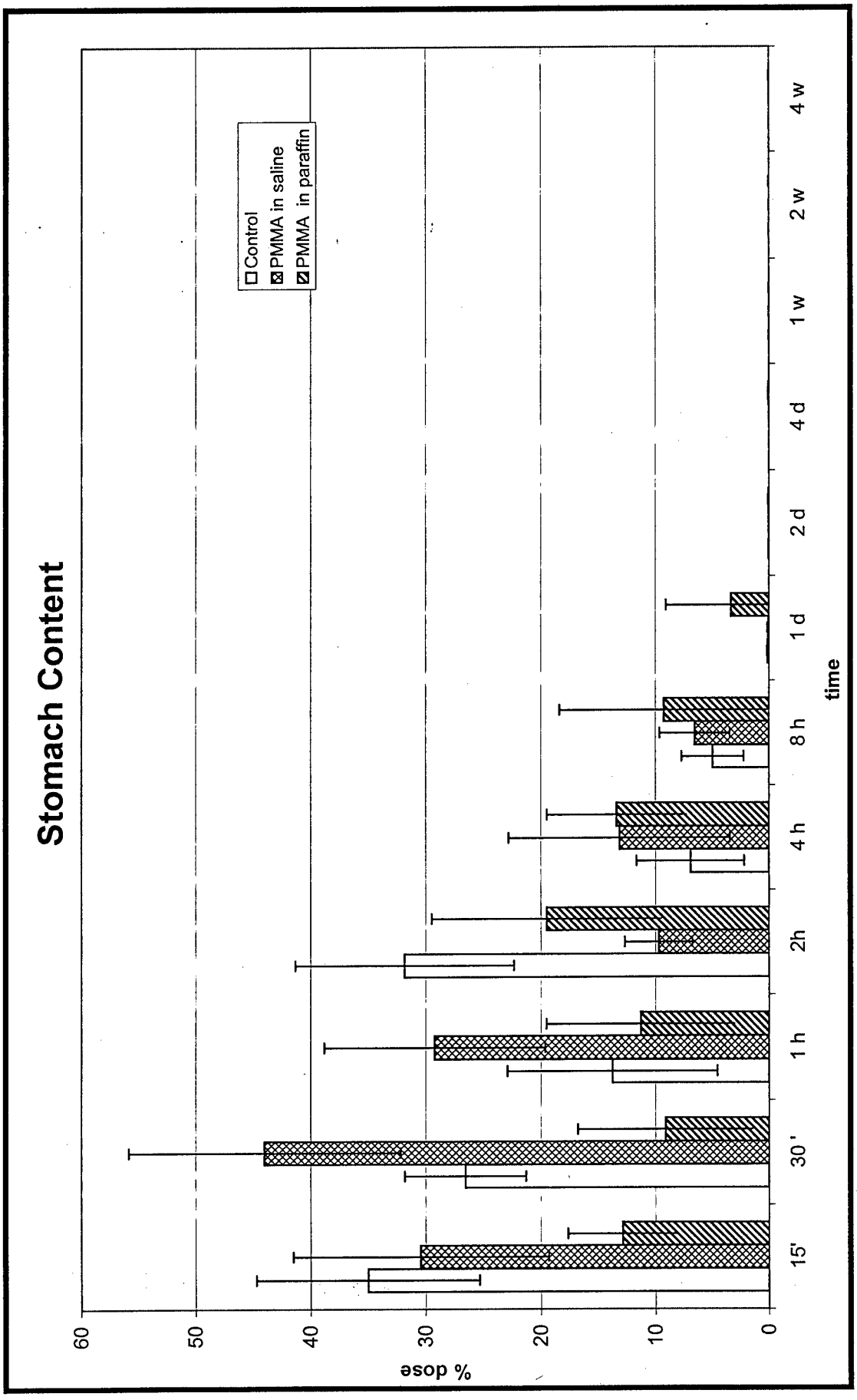


Fig 10: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in stomach walls after oral administration.

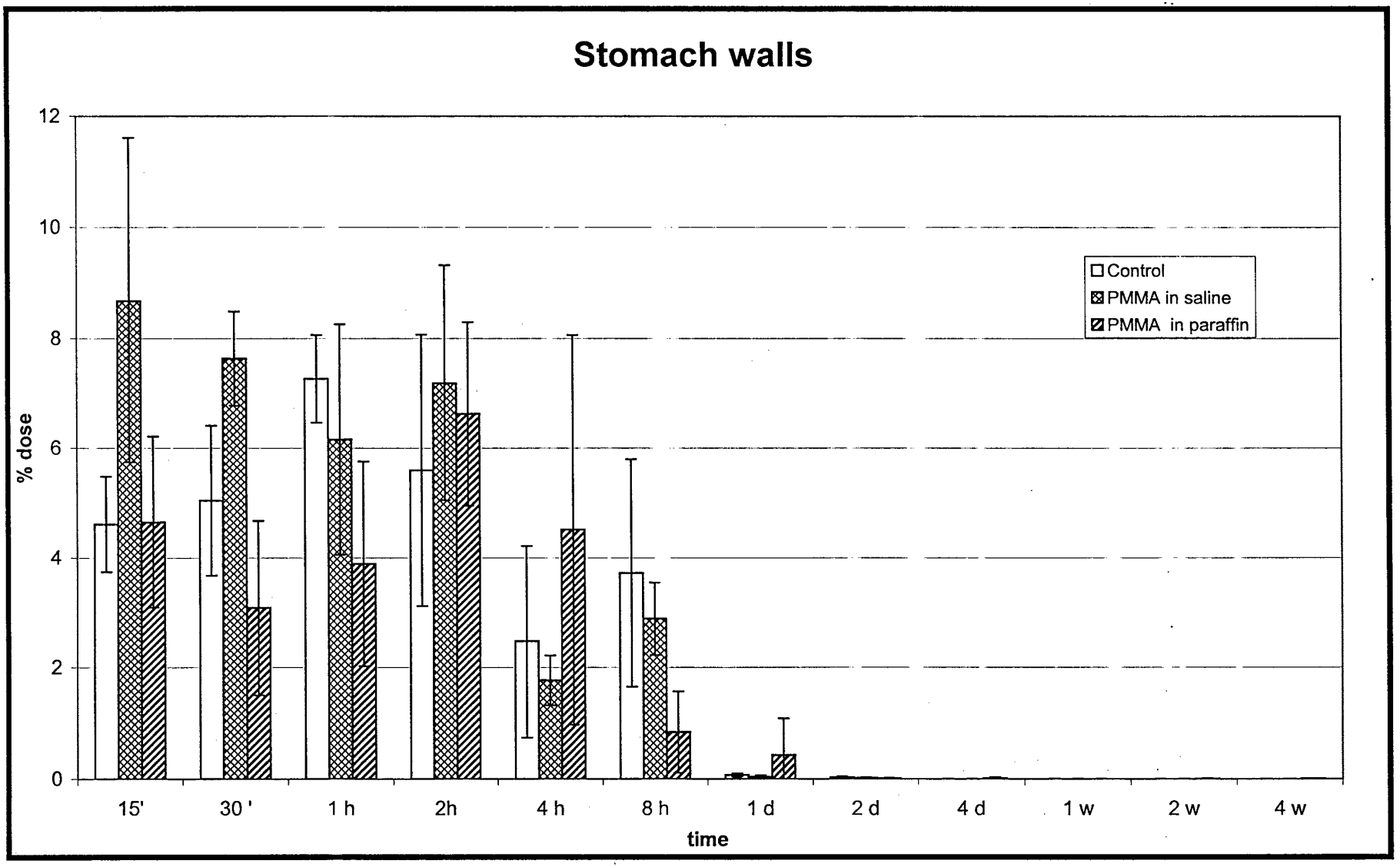


Fig. 11: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in small intestine content after oral administration.

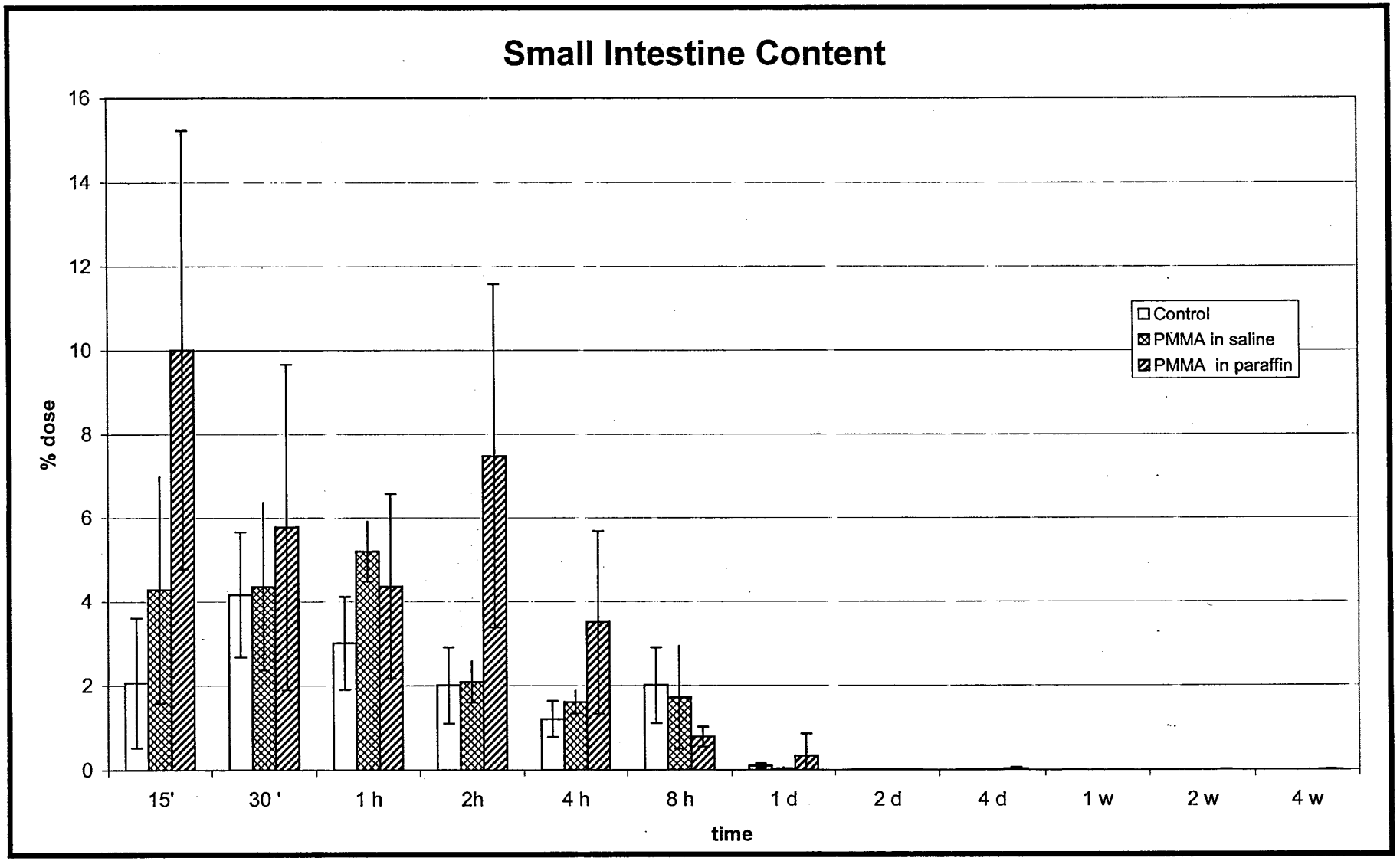


Fig. 12: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the small intestine walls after oral administration.

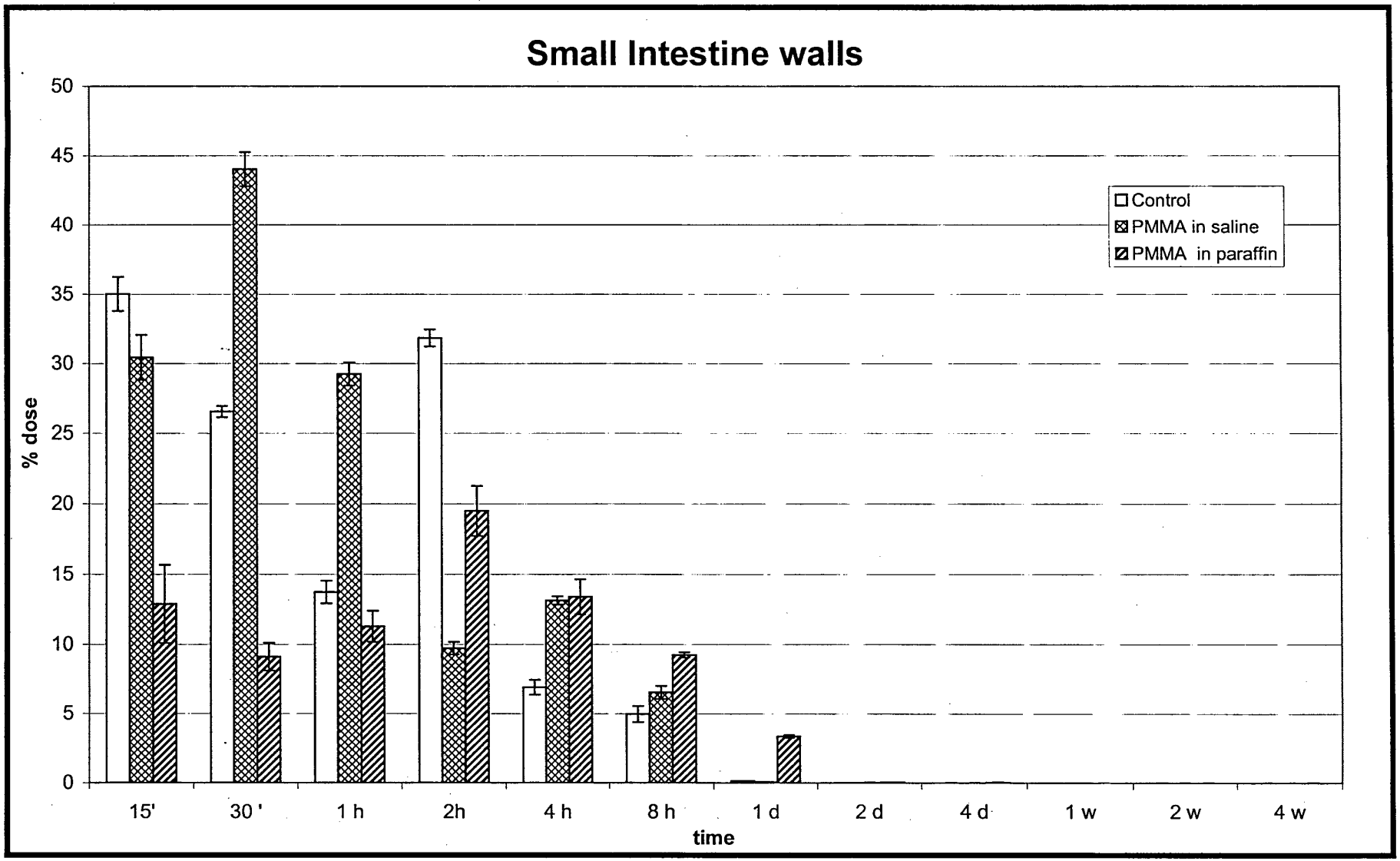


Fig. 13: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the colon content after oral administration.

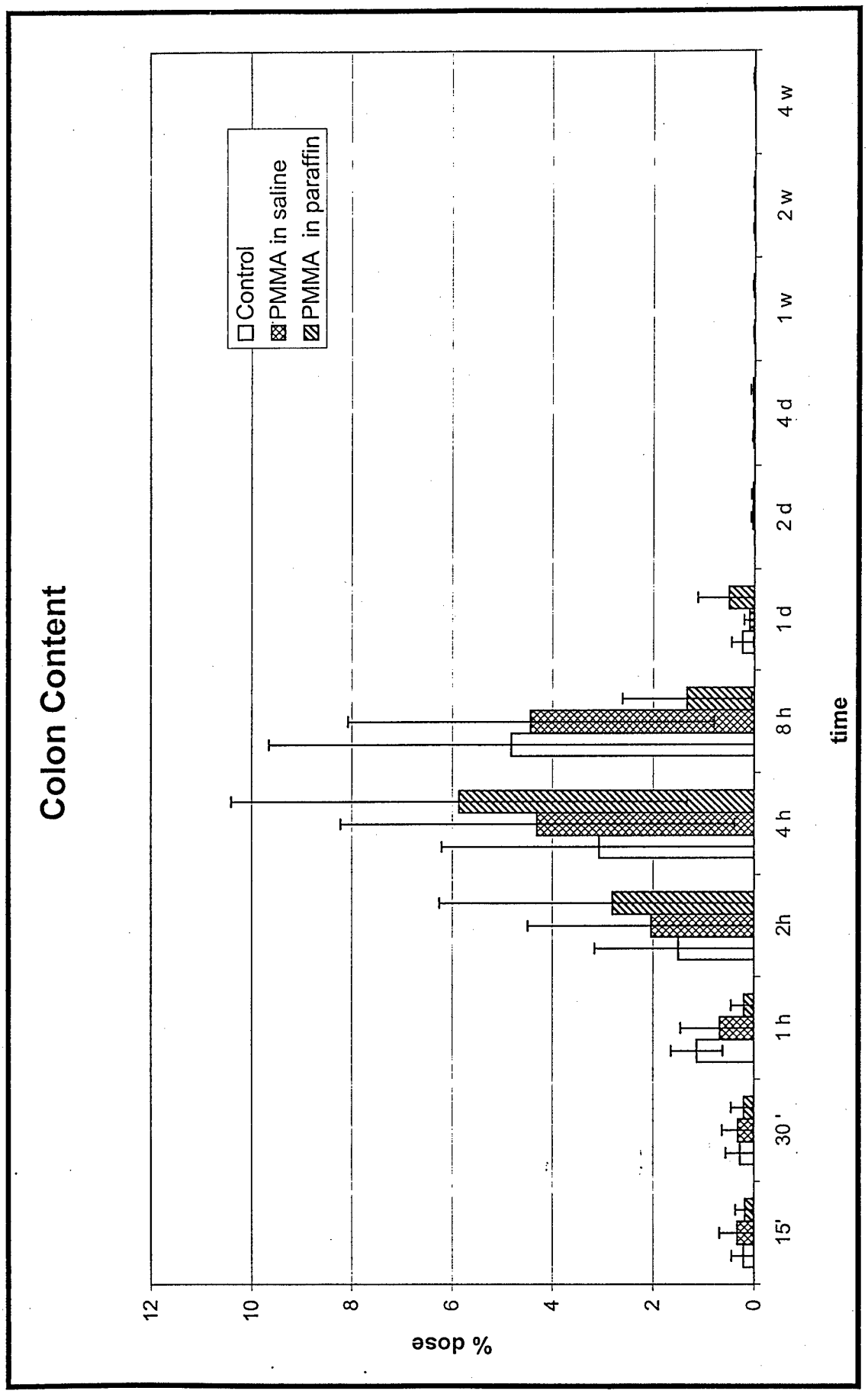


Fig. 14: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the colon walls after oral administration.

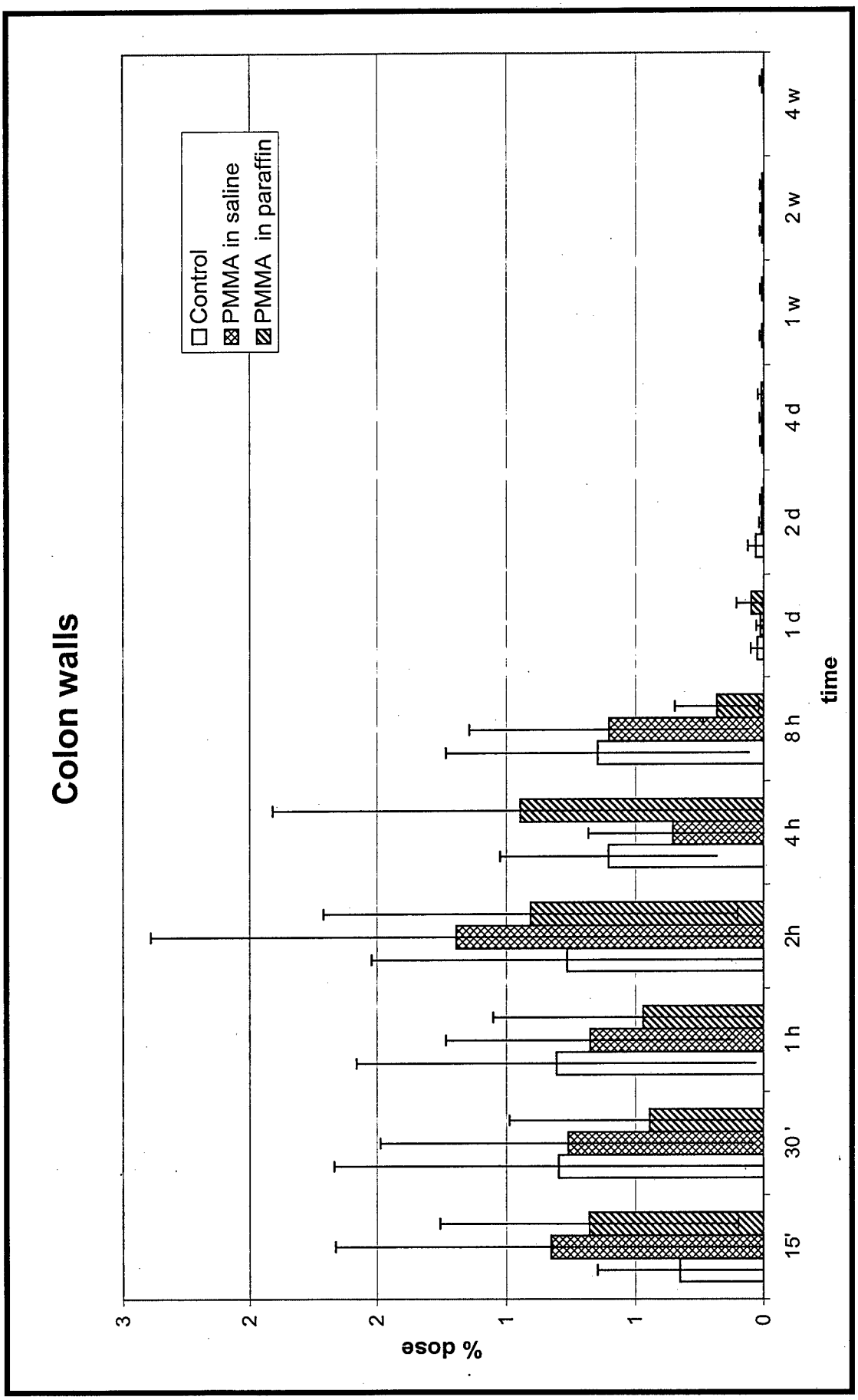


Fig. 15: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in blood after oral administration

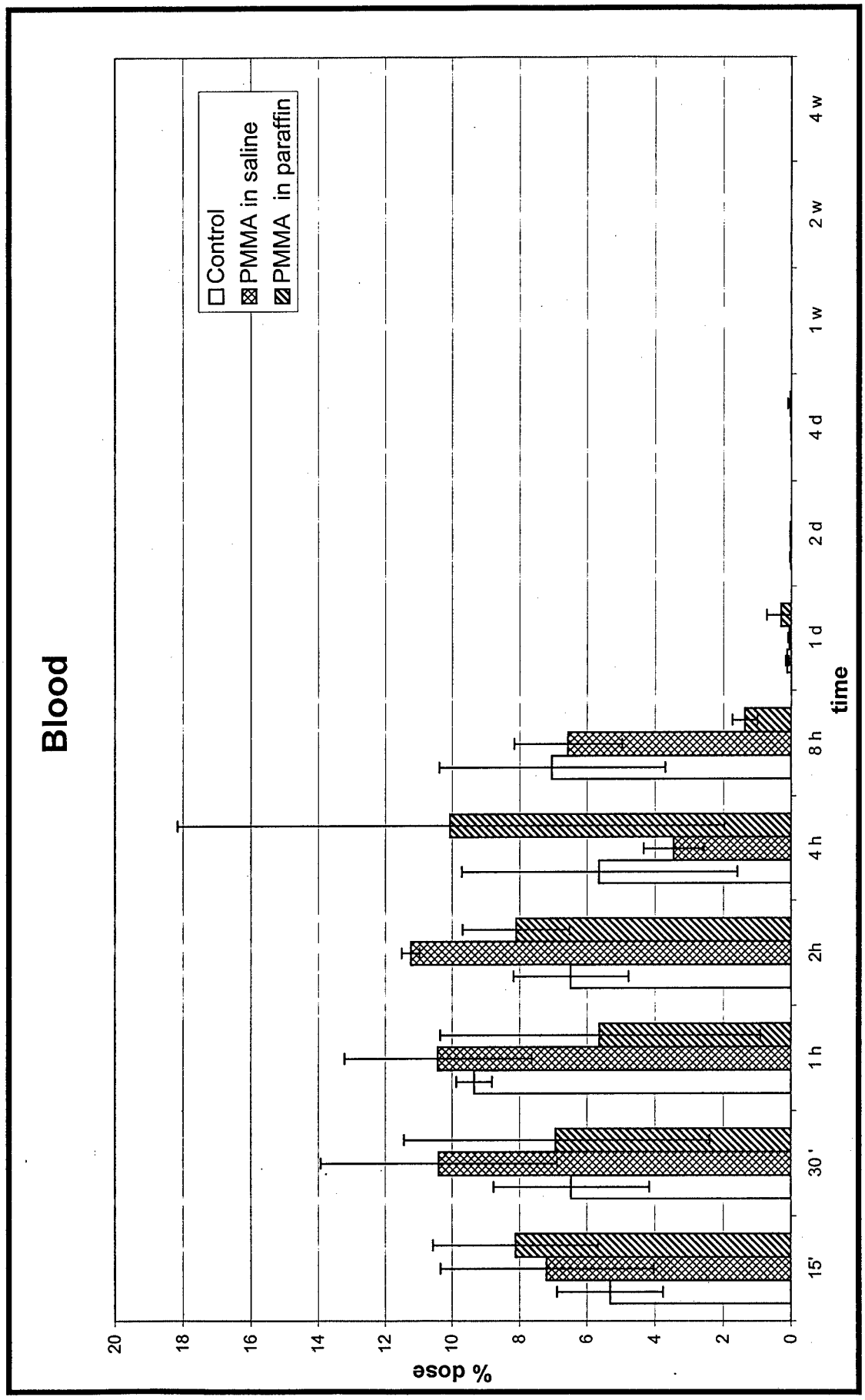


Fig. 16: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the muscles after oral administration

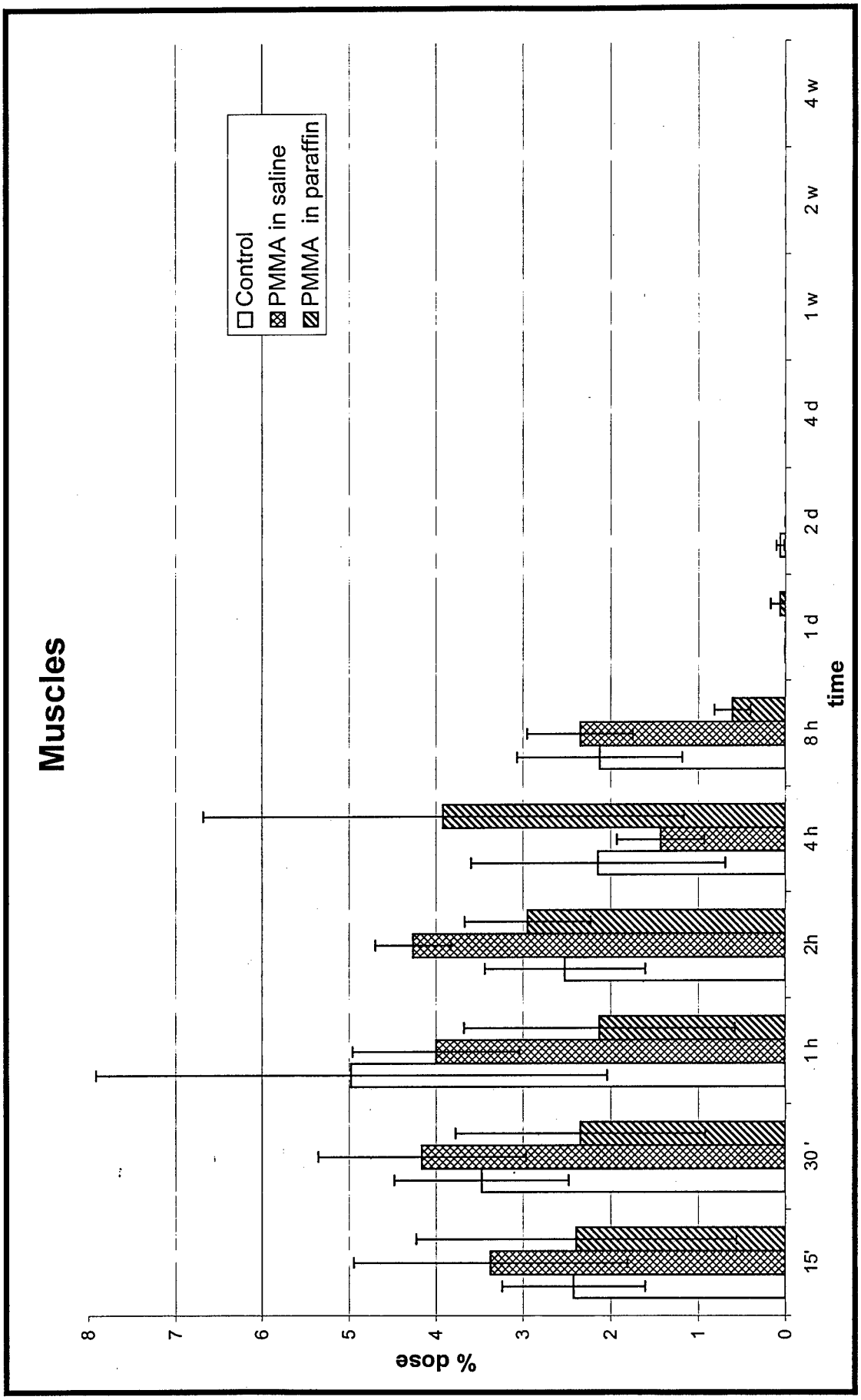


Fig. 17: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in the liver after oral administration

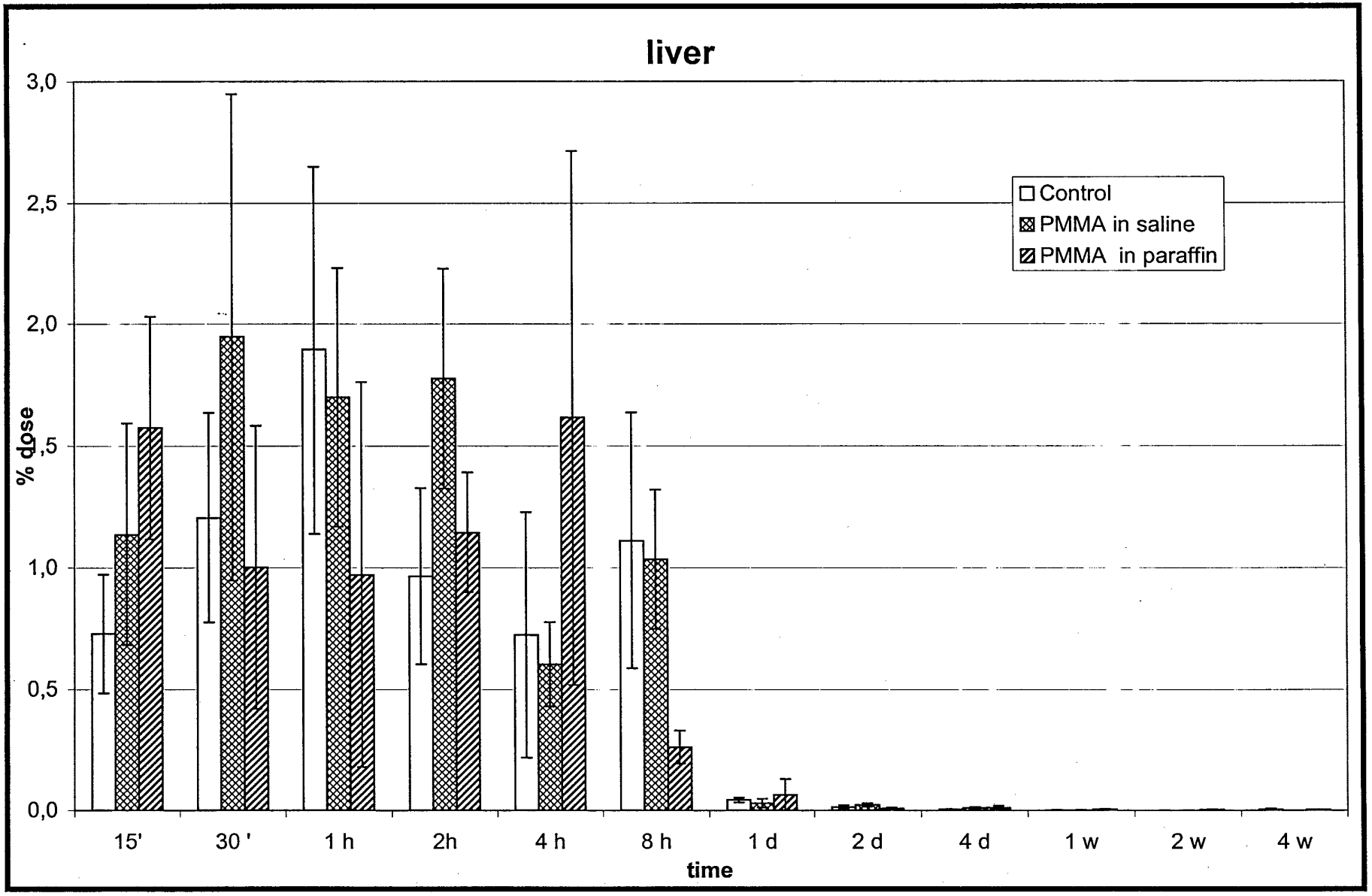


Fig. 18: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the spleen after oral administration.

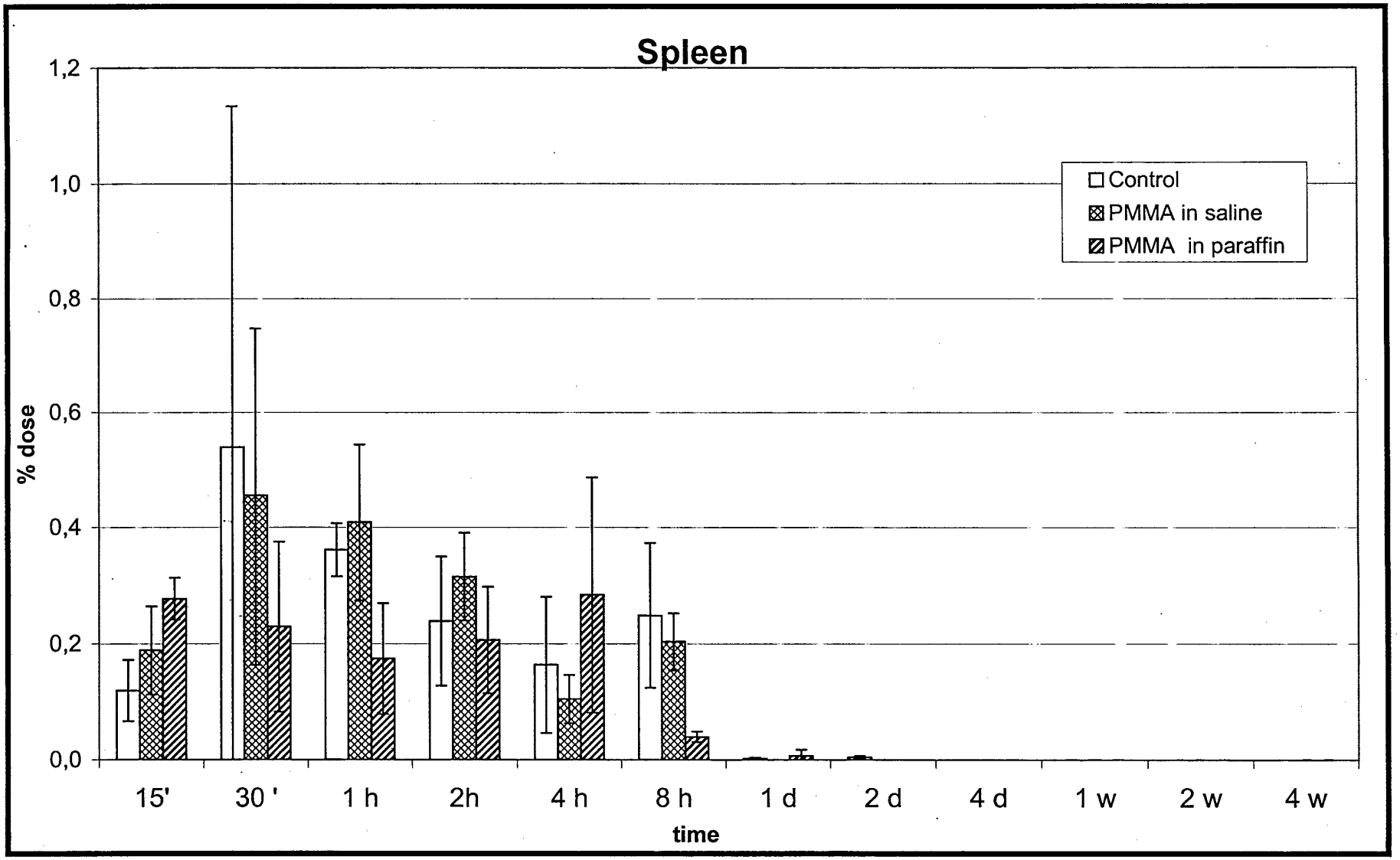


Fig. 19: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the heart after oral administration.

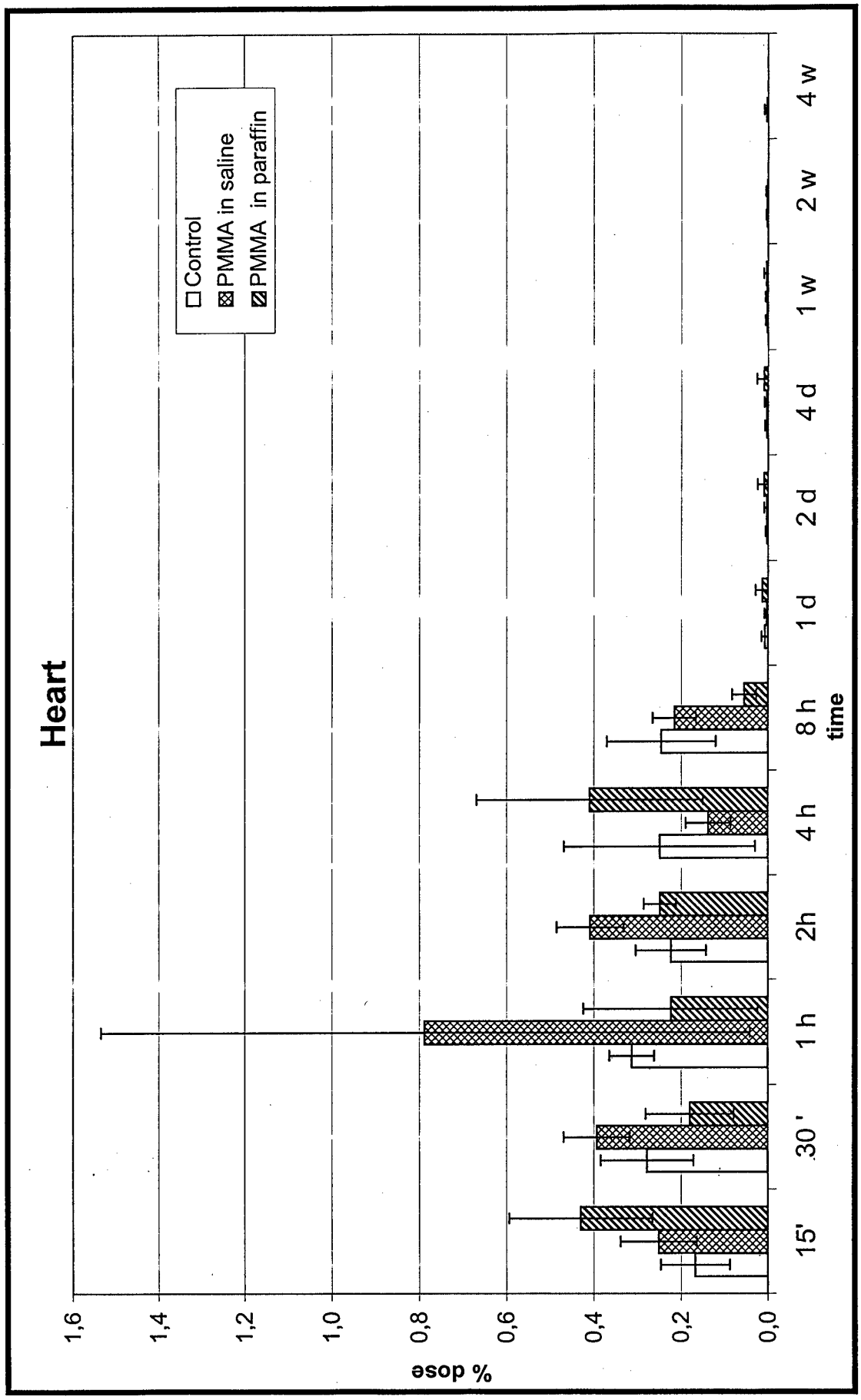


Fig. 20: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or paraffin oil versus time in the lungs after oral administration.

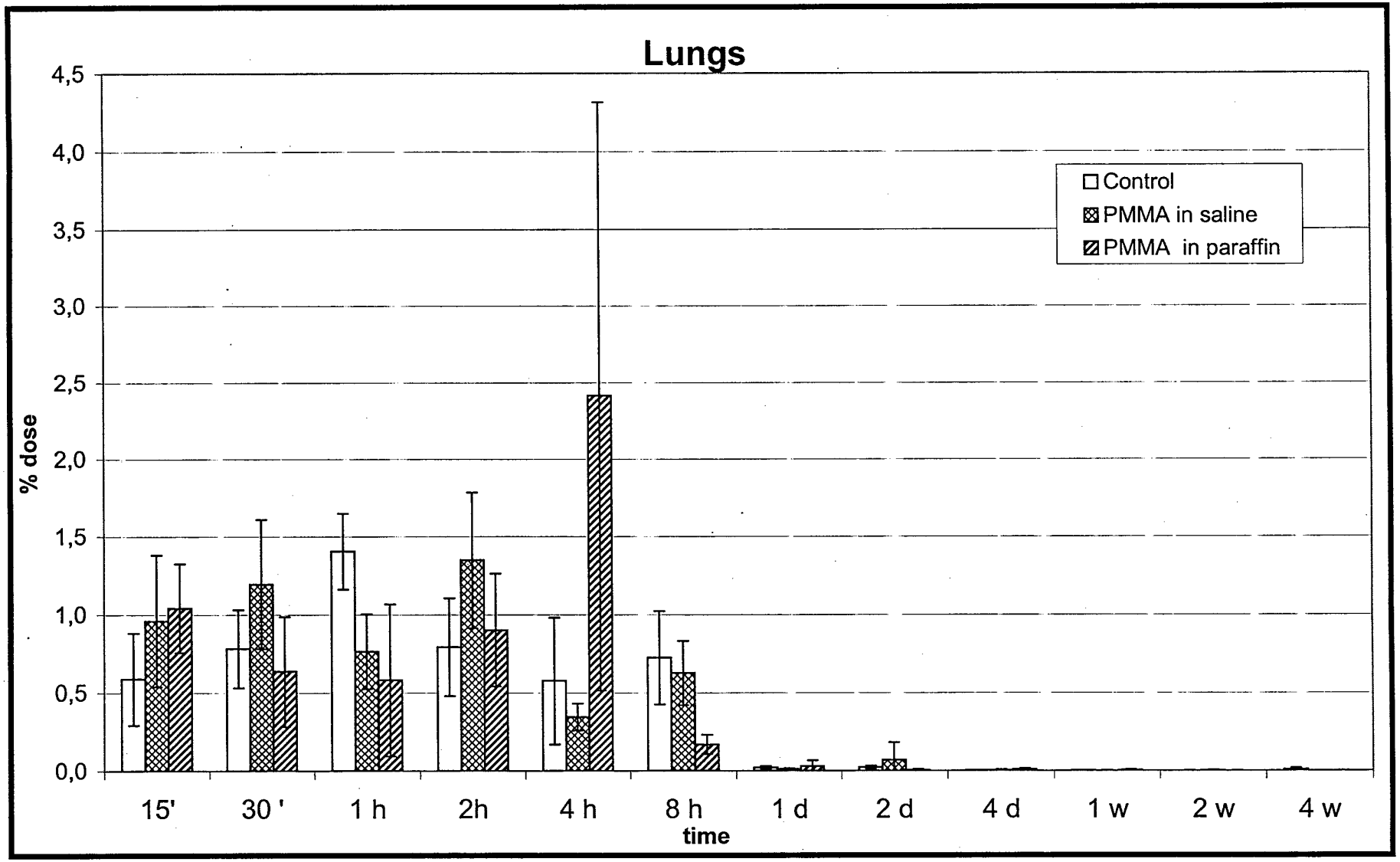


Fig. 21: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in the kidney after oral administration

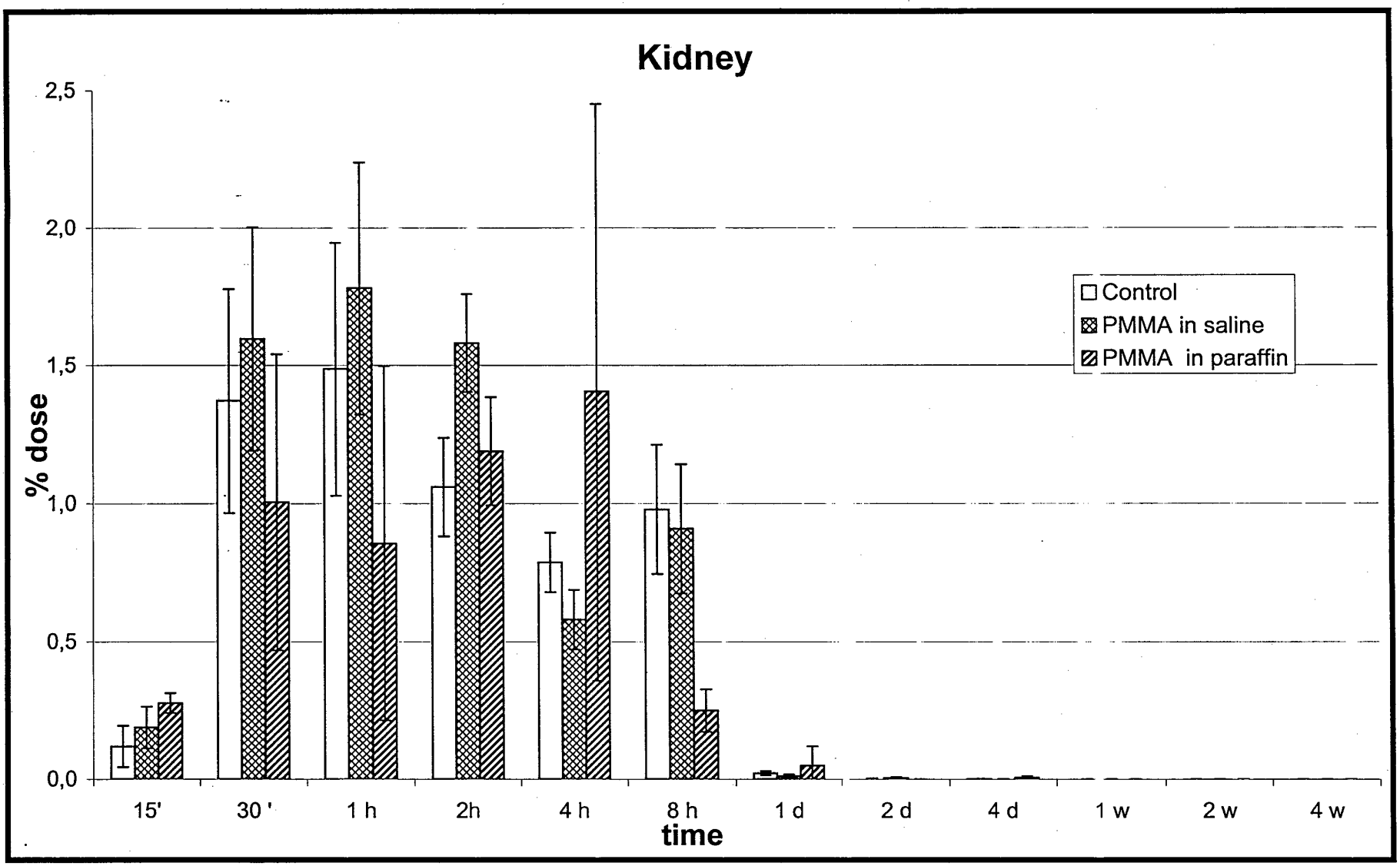
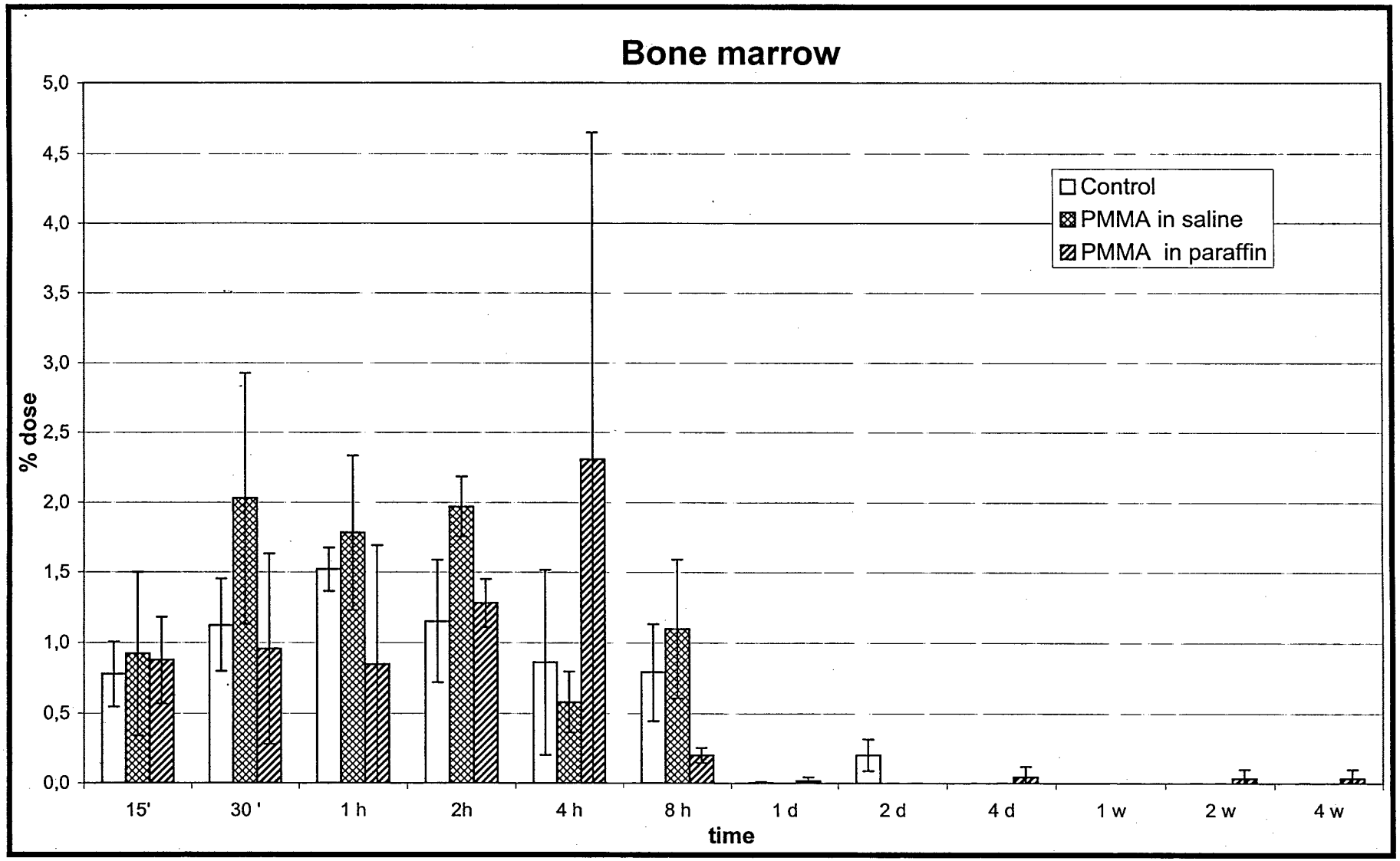


Fig. 22: Percentage of ^{125}I-deglycosilated chain-A ricin solution in saline or adsorbed to PMMA nanoparticles suspended in saline or in paraffin oil versus time in the bone marrow after oral administration.



6.4. Discussion

The results show that deglycosilted chain-A ricin (DGCA) adsorbed to PMMA nanoparticles suspended in water can be used for oral vaccination and yielded some (about 50 %) protection. The very limited vaccination experiments of the present study, however, did not reveal a superior protection compared to the aqueous DGCA vaccine. All other preparations were not able to yield significant protection after oral vaccination.

The most significant and important result of this study is that ELISA antibodies do not correlate at all with protection. Consequently, they can not be used to test or predict the performance of DGCA vaccines, neither after oral after no subcutaneous vaccination.

The whole body autoradiographies showed that DGCA was taken up and distributed through the body to a slightly higher extent with the aqueour PMMA nanoparticle preparation than with the PMMA nanoparticles or the DGCA in water alone. The majority of the radioactivity remained in the gastro-intestinal tract. Nevertheless, as shown by gamma counting, more than 10 % of the administered ^{125}I-label appeared in the blood and was distributed into the residual body. Appart from the blood, the concentrations in the muscles were rather high, i.e. up to 4 % of the administered dose. For most time points between 30 min and 2 hours, the highest concentrations in the blood, muscles and most organs were again obtained with the PMMA aqueous vaccines. These concentrations also were the highest ones for the whole time, supporting the autoradiographic study. However, the differences to the other vaccines were mostly not statistically significant, and after 15 min, 4, and 8 hours the other vaccines, PMMA in paraffine or the aqueous DGCA preparation, yielded higher ^{125}I-label concentrations. After 1 day in all cases very low ^{125}I-levels were obtained. The relatively high blood concentrations after short times, and up to 8 hours and the low levels after 1 day demonstrate the fast elimination of the antigen. Taken together with the relative low differences between the three different vaccine preparations this fast elimination may be an indication that the antigen was at least in part

degraded in the gastro-intestinal tract. The PMMA nanoparticles in the present study performed as well as the aqueous DGCA vaccine without additives in the protection experiments and led to a slightly higher oral absorption of the DGCA antigen. However, the differences between these two prepartations were minimal and probably not relevant. All other preparations yielded no protection and do not warrant further investigation.

7. Key Research Accomplishments

- ELISA antibody titers do not correlate with protection after oral and subcutaneous immunization with deglycosilated chain-A ricin (DGCA).
- Deglycosilated chain-A ricin adsorbed to PMMA nanoparticles suspended in water yields a similar but not significantly better protection than DGCA-solution after oral administration.
- The peroral absorption of ^{125}I-deglycosylated chain-A ricin into the body of mice was slightly higher after adsorption of the DGCA to PMMA nanoparticles and suspension in saline than after their suspension in paraffin or administration of DGCA in form of a solution. Significant body concentrations, however, were only observed for about 8 hours.

8. Reportable outcome

- Ph. D. dissertation of Ms. Letitia Araujo
- A publication in a vaccine journal such as "Vaccine" would be possible provided that the protection and ELISA antibody experiments would be repeated by USAMRIID with at least 20 mice/group using the three experimental groups of the ^{125}I-DGCA studies.

9. Conclusions

Oral vaccination with DGCA in form of a solution or adsorbed to poly(methyl methacrylate) nanoparticles suspended in an aqueous medium leads to an about 50 % protection of mice after an aerosol challenge with ricin. All other nanoparticle products, including suspension in a hydrophobic vehicle (paraffin) or the employment of polylactic acid did not achieve a significant protection. Protection did not correlate at all with ELISA antibodies against ricin after oral as well as subcutaneous vaccination.

Peroral absorption studies with ^{125}I-deglycosilated chain-A ricin using autoradiography as well as gamma counting indicated a slightly I higher but short-lived uptake into the residual body after adsorption of the antigen to PMMA nanoparticles suspended in saline.

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